The role of female hormones and oestrogen receptor status in influencing the anti-tumour effects of zoledronic acid in early breast cancer

Dr Caroline Wilson MBChB, MSc, MRCP

PhD Thesis

Academic Department of Oncology

University of Sheffield

January 2014

Abstract

The AZURE trial was the first adjuvant breast cancer trial to report a novel interaction between the anti-tumour efficacy of zoledronic acid (ZOL) and menopausal status of patients. It showed a significant reduction in disease recurrence events in and outside of bone, in those clinically >5 years postmenopausal, compared to an increase in non-bone recurrences in all others. Data from neoadjuvant studies had also shown an enhanced anti-tumour effect of ZOL in oestrogen receptor negative (ER-ve) tumours. Three main questions arose from these data and were the focus of this thesis; firstly how can postmenopausal status be accurately defined in patients?, secondly, which reproductive hormone is interacting with the drug?, and thirdly, what are the molecular mechanisms?

Follicle stimulating hormone (FSH), oestradiol and inhibin A were evaluated from 806 patients recruited to AZURE. With all 3 hormones in postmenopausal range, ZOL treatment resulted in an improved invasive disease free survival compared to control (HR 0.809, 95%CI 0.537-1.22). No single hormone was predictive of an interaction with ZOL. A low pretreatment FSH and oestradiol were borderline significant for a shorter time to bone and distant recurrence respectively. Evaluation of serum, pre- and post neoadjuvant FEC_{100}chemotherapy +/- ZOL in 40 patients recruited to the ANZAC trial, identified changes in the tumour suppressor activin (↑) and tumour promoter follistatin (↓) in postmenopausal patients and those with ER-ve primary tumours. The direct effect of ZOL on these proteins in breast cancer cell lines *in vitro* and *in vivo,* showed a reduction in follistatin secretion from ER-ve cell lines only, which was diminished in the presence of inhibin A. Moreover, ZOL and inhibin A altered levels of these proteins in the bone microenvironment, with a reduction in bone follistatin levels after ZOL only in ovariectomised mice. In addition, inhibin A decreased bone activin levels.

These data suggest that pretreatment evaluation of reproductive hormones can assist in selection of postmenopausal patients for adjuvant ZOL, and breast cancer cells may preferentially home to premenopausal bone (low FSH), preferring distant non-bone sites in postmenopausal women (low oestradiol). ZOL has novel direct and indirect effects on activin and follistatin levels in the tumour and bone microenvironment that is influenced by ER status of tumor cells and presence of inhibin A.

Dedication

To Mum.

You made me everything that I am today.

You are forever in my thoughts.

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Acknowledgements

My first thank you is to my two supervisors Prof Coleman and Dr Holen. Your patience and guidance over the past 3 years have been immense and I am so very grateful. You both supported my time out of clinical training to experience research full time, an opportunity that I cannot say thank you enough for.

A huge thank-you goes to Dr Holen's lab team in particular Alyson Evans who taught me how to be a clinician in a lab! Also to my colleagues and now firm friends Dr Hannah Brown and Dr Faith Nutter who have been so patient with my constant questions about science and who have guided me with my *in vivo* work. My thanks go to Prof Nicola Brown for allowing my *in vivo* work to be carried out under her project licence. In addition, thanks also must be given to Dr Penny Ottewell and Dr Matthew Winter for their permission to use samples obtained from their research. I would also like to thank Prof Simon Cross for reviewing my histology slides in his free time.

My thanks are also extended to the Leeds Clinical Trials Unit in particular the statasticians Helen Marshall and Samantha Hinsley who have been patient with my requests, and explained complicated trial statistics in a manner I could understand.

To my Dad, thank you for believing in me and supporting me now and always. Thank you to my great friends who have painstakingly proofread chapters for me, and to Chris and Steph for their support. To Macey, thank you for being a constant companion.

Finally thanks must go to all the patients recruited to the AZURE and ANZAC trials who kindly donated their blood for future analysis, without which this research would not have been possible, and to Weston Park Hospital Cancer Charity for funding the research.

Dr Caroline Wilson,
Academic Unit Of Clinical Oncology,
Sheffield University,
January 2014

Published research and awards relating to PhD research

Awards

Best oral presentation - Differential anti-tumour effects of zoledronic acid in breast cancer according to ER status and levels of female hormones**.** Cancer and Bone Society 13th International Conference. November 2013, Miami. Florida.

Grants

Wilson C, Holen I, Coleman RE. Endocrine/paracrine interactions in breast cancer-do they modify the response to zoledronic acid? October 2011. Weston Park Hospital Cancer Charity. **£28,422**

Wilson C, Holen I, Coleman RE. Can the ovarian hormone inhibin A influence the survival of metastatic breast cancer cells. September 2012. Weston Park Hospital Cancer Charity. **£21,223**

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Wilson C, Coleman RE, Winter MC, Ottewell P, Holen I (2013). Differential anti-tumour effects of zoledronic acid in breast cancer according to ER status and levels of female hormones. Abst P104. Cancer and Bone Society 13th International Conference, Miami, Florida.

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RE Coleman, EJ Rathbone, HC Marshall, **C Wilson**, JE Brown, F Gossiel, WM Gregory, D Cameron, and R Bell (2012). Vitamin D, but not bone turnover markers, predict relapse in women with early breast cancer: an AZURE translational study. Cancer Research: Dec 15, 2012; Volume 72, Issue 24,S6-4. SABCS

Oral presentations at international meetings

Wilson C, Coleman RE, Winter MCW, Ottewell P, Holen I (2013). Differential anti-tumour effects of zoledronic acid in breast cancer according to ER status and levels of female hormones. Cancer and Bone Society 13th international Conference. Miami. Florida.

Wilson C. The biology of bone disease/update on current challenges in translational research (2013). Britich Thoracic Oncology Group. London. (Invited speaker).

RE Coleman, EJ Rathbone, HC Marshall, **C Wilson**, JE Brown, F Gossiel, WM Gregory, D Cameron, and R Bell (2012). Vitamin D, but not bone turnover markers, predict relapse in women with early breast cancer: an AZURE translational study. San Antonio Breast Cancer Symposium Texas. December 2012.

Abbreviations

ActRI	Activin receptor type I
ActRII	Activin receptor type II
ALK	Analplastic lymphoma kinase
ASCO	American Society of Clinical Oncology
ATP	Adenosine tri-phosphate
AUC	Area under the cutve
BCA	Bicinchonic acid
BM	Bone marrow
BMD	Bone mineral density
BMI	Body mass index
BMPs	Bone morphogenic proteins
BSA	Bovine serum albumin
BSAP	Bone specific alkaline phosphatase
BV:TV	Bone volume:tissue volume
CDK	Cyclin dependent kinase
CI	Confidence interval
CMF	Cyclophospamide, methotrexate, 5-fluorouracil
CSC	Cancer stem cells
CT	Chemotherapy
CTRU	Clinical Trials Research Unit
CTX	Carboxyterminal telopeptide of type I collagen
CVs	Coefficients of variability
DAB	3,3’-diaminobenzidine
DAPI	4’6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DiD	1,1’-Dioctadecyl-‘, 3’-Tetramethyllindodicarbocyanine, 4-Chlorobenzenesulfonate
DFS	Disease free survival
DKK-1	Dickopf-related-protein-1
DMEC	Data monitoring committee
DMEM	Dilbecco’s Modified Eagle Medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTCs	Disseminated tumour cells
ECL	Electrochemiluminescent
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoabsorbance assay
ER-ve	Oestrogen receptor negative
ER+ve	Oestrogen receptor positive
ESC	Endothelial stem cells
FCS	Fetal calf serum
FEC	5-Flurouracil, Epirubicin, Cyclophosphamide
FLRG	Follistatin related gene
FMP	Final Menstrual period
FPP synthase	Farnesyl pyrophosphate synthase
FSH	Follicle stimulating hormone
GGOH	Geranylgeraniol
GnRH	Gonadotrophin releasing hormone
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HEPES	Hydroxyethyl piperazineerthanesulfonic acid
HER2	Herceptin -2-receptor
HPG	Hypothalamic pituitary gonadal
HR	Hazard ratio
HSC	Haematopoetic stem cells
IDC	Invasive ductal carcinoma
IDFS	Invasive disease free survival
IHC	Immunohistochemistry
IP	Intraperitoneal
IQR	Interquartile range
IU/l	International units per litre
IV	Intravenous
LH	Lutenising hormone
LMP	Last menstrual period

MH1	MAD homology 1
MH2	MAD homology 2
MMPs	Matrix metalloproteins
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
N.Ob/mm	Number of osteoblasts per mm bone
N.Oc/mm	Number of osteoclasts per mm bone
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NIBSC	National Institute for Biological Standards and Controls
NTX	N-telopeptide of type I collagen
Ob	Osteoblast
Oc	Osteoclast
OPG	Osteoprotegerin
OS	Overall survival
OVX	Ovariectomy
P1NP	Procollagen type 1 amino-terminal propeptide
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween
pCR	Pathological complete response
PFA	Paraformaldehyde
pSmad2C	Phosphorylated Smad2 COOH tail
pSmad2L	Phosphorylated Smad2 linker region
PTH	Parathyroid hormone
PTHrP	Parathyroid related protein
PVDF	Polyvinylidene difluoride membrane
q3-4 wk	Every 3-4 weeks
RANK	Receptor Activator of Nuclear factor kβ
RAS	Rat sarcoma
RIA	Radioimmunoassay
RITS	Residual Invasive Tumour Size
ROC	Receiver operating characteristic
ROI	Region of interest

RPMI	Roswell Park Memorial Institute medium
SDS	Sodium docecyl sulfate
SEM	Standard error of mean
Smad	Mothers Against Decapentaplegic homolog
SREs	Skeletal related event
ST	Standard therapy
STH	Sheffield Teaching Hospitals
TGFβ	Transforming growth factor β
TGFβRII	Transforming growth factor receptor type II
TMB	Tetramethylbenzidine
TNM	Tumour, nodes, metastases
TRAP	Tartrate resistant acid phosphatase
TSC	Trial steering committee
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
ZOL	Zoledronic acid
μCT	Micro-computed tomography

Chapter 1. Introduction.

1.1 Breast cancer- tumour spread is an early phenomenon.

1.1.1 Breast cancer - burden of disease.

Breast cancer is the most common cancer in women, with ~50,000 new cases of breast cancer diagnosed in the UK in 2010. Over 80% of all diagnoses are made in women over the age of 50. Women in the UK now have a lifetime risk of 1 in 8 for the development of breast cancer (CRUK 2013). Outcomes for these women depend on the stage of the disease at presentation. The concept of 'stage' includes an assessment of established adverse prognostic factors including size of breast tumour, involvement of local lymph nodes and spread to distant organs. Advancing stage includes an increase in the size of the primary tumour, increased involvement of lymph nodes and local structures with cancer, and stage IV involves spread outside of the breast to distant organs (Table 1.1). Survival correlates with stage of disease and 5 year survival rates are 90% for stage 1, 70% stage II, 50% stage III and 13% stage IV. Reassuringly, data from Cancer Research UK showed that in over 17,000 women diagnosed with breast cancer in 2006-2009, the majority presented with stage I or II disease, with <10% stage III and only 5% stage IV (CRUK 2013). However, even in early stage disease, tumour cells may have spread from the primary and their survival in distant sites will depend on intrinsic tumour factors and also factors in the microenvironments in which they locate.

1.1.2 Dissemination of tumour cells in early breast cancer.

Breast carcinomas occur when normal breast epithelial cells develop somatic mutations that are not effectively removed by DNA repair mechanisms, resulting in accumulation of mutations in daughter cells. Over time, often decades, these mutations accumulate and the cells ultimately change from normal epithelial cells to invasive carcinoma (Weinberg 2007). This clonal model of tumour progression indicated that over time, cancers would develop sufficient genetic aberrations that promoted not only their survival, but also their ability to migrate through local tissues and seed to and survive in distant organs.

Table 1.1. Stage of breast cancer at diagnosis according to tumour size and spread to local and distant structures.

Stage	Description
I	
A	Tumour<2cm and no spread outside breast
B	Tumour<2cm with tumour cells detectable in local lymph nodes
II	
A	Tumour <2cm and in 1-3 lymph nodes Tumour 2-5cm no lymph nodes involved
B	Tumour 2-5cm with tumour cells detectable in lymph nodes Tumour 2-5cm and in 1-3 lymph nodes Tumour >5cm no lymph nodes involved
III	
A	Tumour any size and in 4-9 lymph nodes Tumour >5cm with tumour cells detectable in lymph nodes Tumour >5cm and in 1-3 lymph nodes
B	Tumour spread to skin or chest wall and <9 lymph nodes
	Tumour has spread to skin and chest wall and >10 lymph nodes or to distant lymph nodes ie collar bone or breast bone
IV	Tumour any size with spread to distant organs

Table adapted from TNM staging of breast cancer. CRUK website. www.cancerresearchuk.org

However, challenges to this theory include the observation that disseminated tumour cells from the bone marrow of breast cancer patients without established metastatic disease, have fewer genetic mutations than the primary tumour (Schmidt-Kittler, Ragg *et al*. 2003). In addition, a series of experiments using fluorescent-activated cell sorting (FACS), identified a sub-population of breast tumour cells that were more tumorigenic than the bulk of the tumour mass. These cells were positive (+ve) for CD44 on their cell surface, and when injected sub-cutaneously into NOD/SCID mice, formed tumour colonies when only 100 cells were injected compared to >20,000 of the low CD44 expressing tumour cells that did not form tumours. The tumours that formed from the CD44+ve cells contained a mixed population of CD44+ve and –ve cells (Al-Hajj, Wicha *et al*. 2003). These data suggested that a small minority of cancer 'stem' like cells were responsible for forming the bulk of the tumor cells, and that the majority of the tumour cells lack proliferative potential. It therefore is plausible that within very early breast tumours, possibly before clinical detection, there will be a sub-population of cancer stem cells (CSC), which have the capacity to proliferate and form new tumour colonies at distant sites. CSC have been shown to be more resistant to commonly used anti-cancer therapies, including radiotherapy and chemotherapy (Pinto, Widodo *et al*. 2013), and overexpression of CD44 in MCF7 cells confers resistance to endocrine therapy with tamoxifen (Hiscox, Baruha *et al*. 2012). These data would explain, in part, why early breast cancers treated with multi-modality curative treatment including surgery, radiotherapy and chemotherapy can still recur, sometimes decades after completion of 'curative' treatment.

It has been accepted for over 125 years that certain tumours seed to specific distant sites, as first described by Stephen Paget in 1889 (Paget 1889). Breast cancer often spreads to bone in a process involving breakdown and migration through stroma, followed by intravasation and avoidance of host immune system, and ultimately extravasation and survival in bone (Suva, Washam *et al*. 2011) (Fig 1.1). However, only 0.01% of the tumour cells that enter the circulation will form tumour colonies in bone. Expression of receptors on breast cancer cells such as CXCR4, have been identified as promoting a 'bone homing' tumour cell phenotype. CXCR4 mediates homing to organs that express high levels of its ligand CXCL12, such as bone (Allinen, Beroukhim *et al*. 2004).

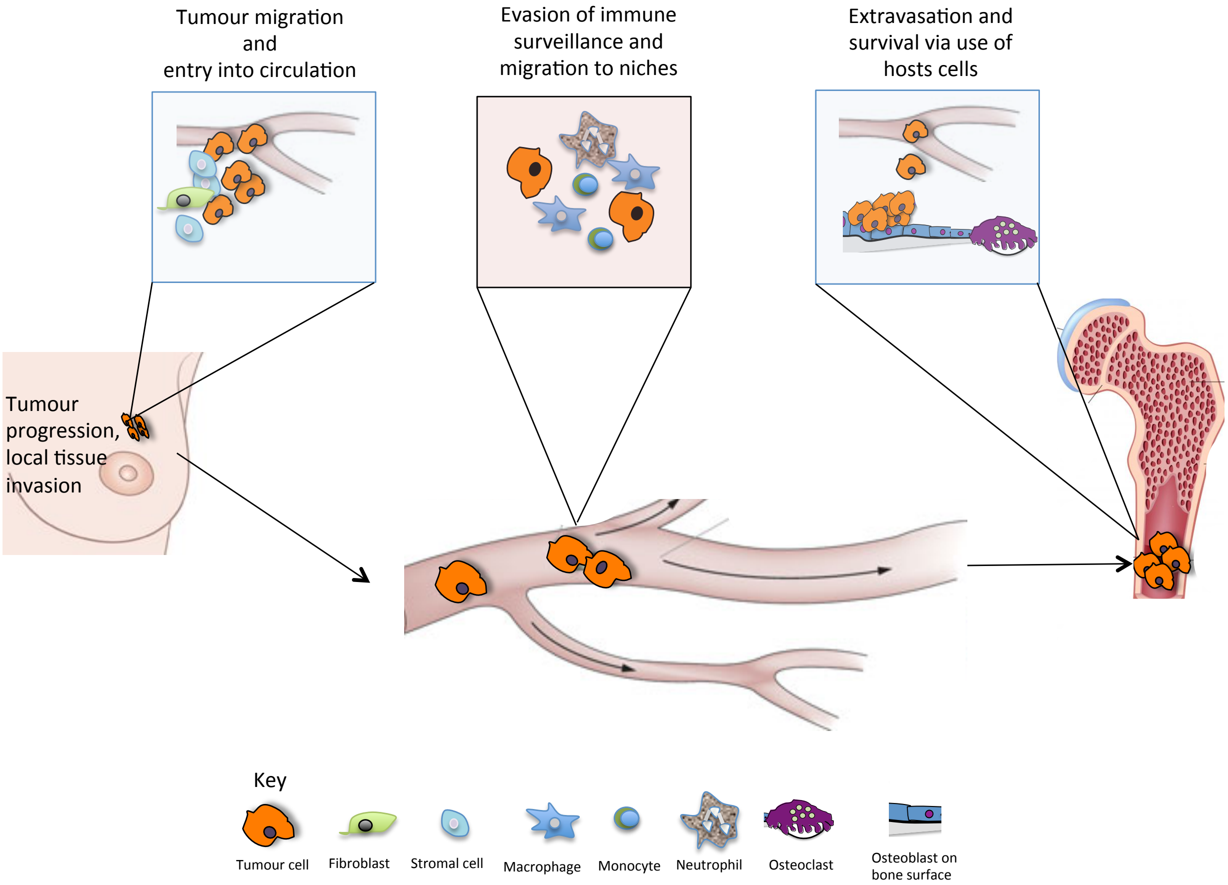


Figure 1.1. Process of spread of breast cancer from primary site to bone.

This multistep process involves local tumour progression and stromal invasion, followed by intravasation and evasion of host immune system and survival of shear forces within the circulation and ultimately extravasation at the metastatic site with utilisation of host stromal cells at this new site to assist survival. <0.01% of cells that enter the circulation will be able to form bone metastases and these cells may have stem cell like properties rendering them more able to survive and proliferate.

Gene signature studies on primary tumours have not identified a single gene or genetic signature that predict if a primary breast tumour will form bone metastasis (Holen 2012), therefore although the intrinsic properties of the cancer cells will play a key role in promoting survival and spread, environmental factors at distant sites such as bone, may also influence survival and growth of tumour cells.

1.1.3 Host stromal cells affect tumour cell survival in both the primary and bone microenvironment.

It is well established that stromal cells pay a key role in modification of tumour cell survival, migration and proliferation, both in the primary tumour and in distant/secondary tumour microenvironments. The primary breast tumour comprises multiple cell types including tumour cells, fibroblasts, macrophages, stromal cells, neutrophils, blood vessels and extracellular matrix (Psaila, Kaplan *et al*. 2006) which all play a role in facilitating or hindering tumor cell proliferation and migration (Fig 1.2). Cancer associated fibroblasts promote tumour growth and angiogenesis when injected with MCF-7 breast cancer cells *in vivo* (Orimo, Gupta *et al*. 2005), and tumour associated macrophages assist neovascularisation by increasing tumour secretion of vascular endothelial growth factor (VEGF) (Bingle, Lewis *et al*. 2006), and tumour invasiveness by secretion of matrix metalloproteins (MMPs) leading to break down of stroma (Bingle, Brown *et al*. 2002).

In addition, primary tumours contain bone-derived cells including haematopoetic stem cells (HSC) and endothelial stem cells (ESC) allowing communication between the primary tumour and bone (Barcellos-de-Souza, Gori *et al*. 2013). These HSC are thought to ‘prime’ future metastatic sites, and chemokines secreted from the primary tumours can influence the location of these cells therefore controlling the site of future metastases. This was evidenced *in vivo* by intradermal injection of Lewis lung cancer cells into C57Bl/6 mice that were treated with daily intraperitoneal conditioned medium from B16 melanoma cells or serum free medium starting 2 days prior to tumour cell injection. Animals treated with conditioned medium developed a metastatic profile more characteristic of melanoma rather than the Lewis lung cancer cells (Kaplan, Riba *et al*. 2005). This concept of a ‘premetastatic niche’ remains to be confirmed in bone.

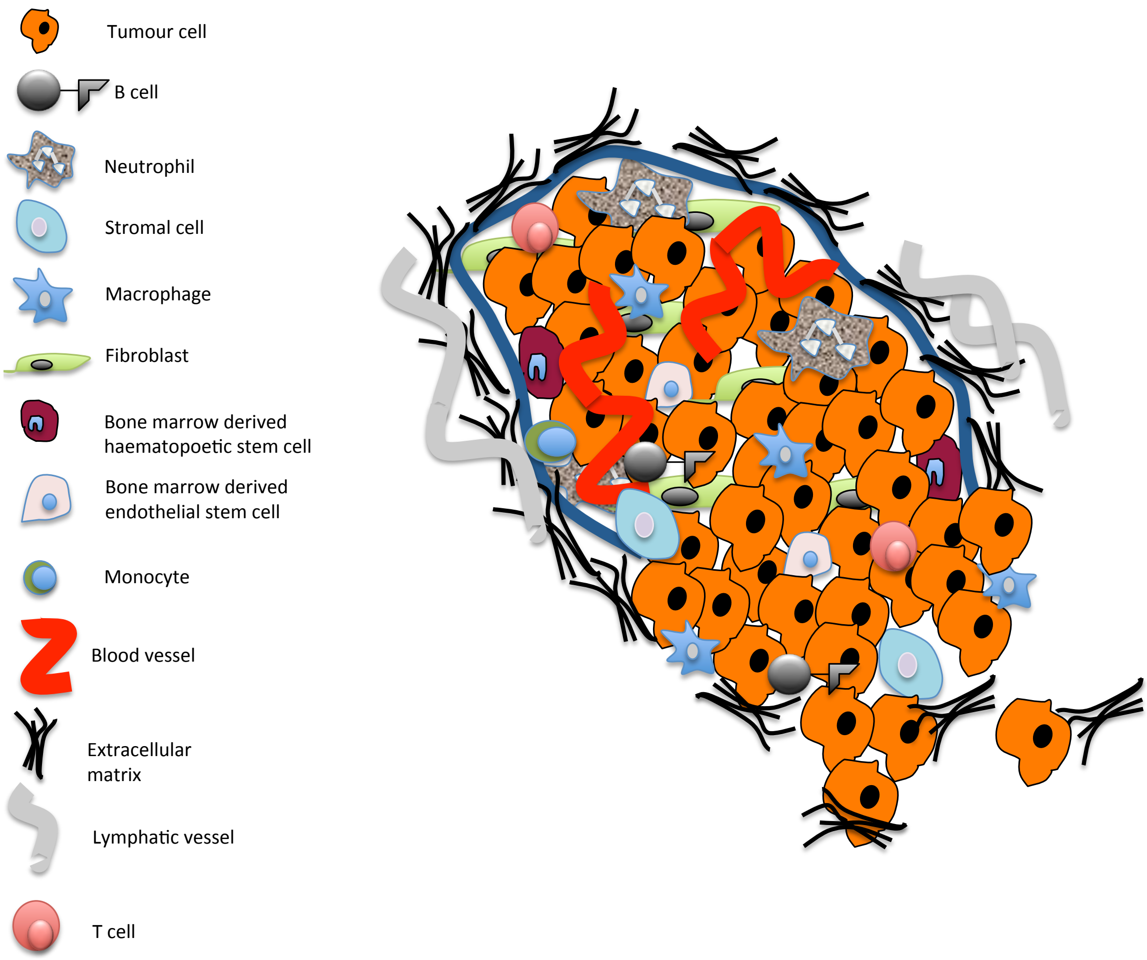


Figure 1.2 The microenvironment of the primary breast tumour

The microenvironment contains a plethora of host cells, in addition to tumour cells, which can modify tumour cell survival, proliferation and local invasion.

Once breast cancer cells home to bone, they localise to areas within the bone that are highly vascularised such as trabecular bone (Holen 2012), where they interact with the host bone cells. In addition to the stromal cells already described, the unique bone microenvironment includes the bone forming osteoblasts and the bone resorbing osteoclasts. Osteoclasts are large multinucleated cells derived from haematopoietic progenitor cells that, upon contact with the bone surface, secrete proteolytic enzymes such as cathepsin K and $H^+ Cl^-$ that degrade the bone matrix. Osteoblasts are derived from mesenchymal progenitor cells and lay down new unmineralised matrix in the resorption pits made by osteoclasts (Boyce 2012). The coupling of bone formation and resorption is critical to prevent pathological excess of either process that could alter bone volume and architecture. One such coupling mechanism is Receptor Activator of Nuclear factor κβ (RANK):osteoprotegerin (OPG) system; RANK ligand is secreted by osteoblasts and binds to RANK on the surface of pre-osteoclasts to promote differentiation, this is prevented by osteoblast secretion of the RANK ligand soluble decoy ligand OPG, thus creating an on:off mechanism by which osteoblasts can control osteoclast activity (Dougall, Glaccum *et al.* 1999; Kong, Yoshida *et al.* 1999). Denosumab, a monocloncal antibody against RANK ligand has recently been approved for therapeutic use in bone metastases from breast and other solid tumours.

When tumour cells have developed autonomous cell growth in bone, following acquisition of an adequate blood supply, they form small colonies and secrete factors that affect both bone cell types and disrupt the normal coupling of formation to resorption. Osteoclasts are stimulated by tumour secreted parathyroid-related protein (PTHrP), which increases local production of RANK ligand (Kingsley, Fournier *et al.* 2007). The increase in osteoclast bone resorption releases growth factors such as transforming growth factor β (TGFβ) which are stored in bone matrix which further increase tumour secretion of PTHrP (Mourskaia, Dong *et al.* 2009). Other tumour secreted factors such as bone morphogenetic proteins (BMPs), activate osteoblasts directly which subsequently release more RANK ligand to initiate osteoclast differentiation (Dai, Keller *et al.* 2005). These interactions create what is termed as ‘the vicious cycle’ of bone metastasis (Mundy 1997), a tumour induced self-propagating breakdown of bone.

These changes represent the end of a spectrum of interactions between bone cells and tumour cells, However, the interaction of tumour cells with osteoblasts and osteoclasts in early stages of spread to bone, when tumour cells lack autonomous growth, is yet to be determined. Recent evidence has shown that breast tumour cells that localise to bone after intracardiac injection *in vivo* have early effects on osteoclasts and osteoblasts that are in direct contact with the tumour cells, increasing the numbers of the former and decreasing the numbers of the latter. These changes occurred before lytic lesions were evident on imaging, suggesting that tumour cells can modify bone cells from an early stage (Brown, Ottewell *et al*. 2012). These early interactions may prove to be critical in determining tumour cell fate, either elimination or survival in a dormant state prior to expansion and proliferation either in bone or in other metastatic sites.

1.1.4 Dormancy and quiescence of disseminated tumour cells in bone

On arrival in bone, tumour cells interact with bone stromal marrow cells to colonise a niche in which they are attached and can survive. Bone marrow stromal cells including osteoblasts, osteoclasts, fibroblasts and adipocytes, regulate self-renewal, proliferation and migration of hematopoetic and mesenchymal stem cells under normal physiological conditions (Kollet, Dar *et al*. 2006; Miura, Gao *et al*. 2006). HSCs bind to osteoblasts *in vitro* (Crean, Meneski *et al*. 2004) and it is thought that they need to be in direct contact with osteoblasts to ensure survival (Shiozawa, Havens *et al*. 2008) (Fig 1.3). It is possible that disseminated tumor cells may localise to the HSC and MSC niche and use the same stromal adhesion and environmental signals to enable them to survive. It has been shown that tumour cell lines with bone homing properties express the receptor for annexin II that osteoblasts use to adhere to hematopoetic stem cells, and knockdown of this receptor in prostate cells prevented bone metastasis after intacardiac injection (Shiozawa, Havens *et al*. 2008). Others have shown that expanding the osteoblast niche with the use of PTH, increased the number of disseminated tumour cells in bone from sub-cutaneous prostate tumours *in vivo* (Shiozawa, Pedersen *et al*. 2011). Additional receptor-ligand interactions involve the Notch-Jagged pair; bone homing tumour cells overexpress Jagged which binds to Notch expressed by osteoblasts and osteoclasts facilitating tumour cell invasion and growth in bone (Sethi, Dai *et al*. 2011).

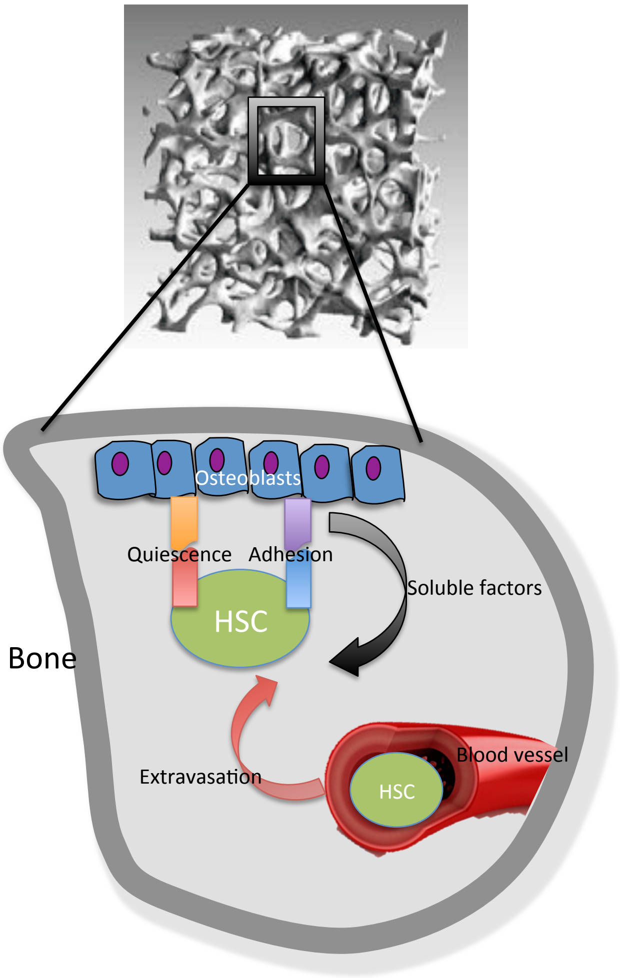


Figure 1.3. The hematopoetic stem cell niche

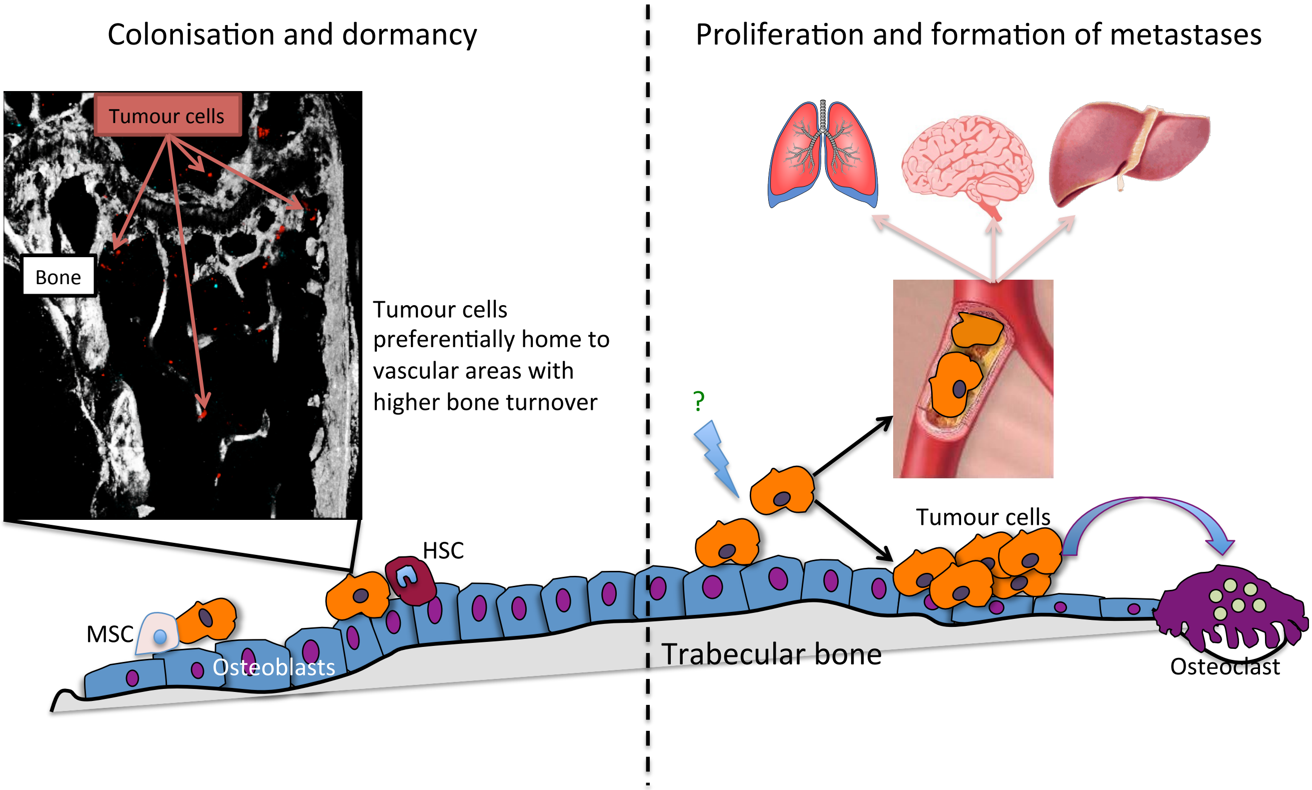
Hematopoetic stem cells (HSC) migrate out of the blood stream and adhere to osteoblasts via ligand receptor interactions such as the adhesion Jagged-Notch receptor. Osteoblasts play an important role in regulation of HSC via secretion of soluble factors, which control proliferation and maintain quiescence. After changes in environmental signals, activation of HSCs occurs, which then re-enter the circulation to bring about their effector functions in other organs.

After attaching to bone, the tumour cells are under the influence of local environmental factors that may be very different to those of the primary site, and can induce stress signals in the tumour cells that facilitate a state of quiescence/dormancy (Wikman, Vessella *et al*. 2008). These cells are not dividing and show cell cycle arrest, however, this state is not permanent and they can revert to a dividing phenotype under activating factors that are yet to be identified (Vessella, Pantel *et al*. 2007). This could subsequently result in development of established bone metastases or the cells may re-enter the circulation and seed to other distant organs (Shiozawa, Havens *et al*. 2008) (Fig 1.4).

1.1.5 Clinical implications of breast tumour growth in bone - the need for prevention.

Once bone metastases are identified radiologically or clinically, the disease is incurable with a median survival of 2 years following clinical diagnosis of bone metastases (Coleman 2005). The impact of the 'vicious cycle' on patients is profound; breast cancer cells in bone cause lytic bone destruction, which leads to skeletal related events (SREs) including hypercalcaemia, pathological fracture, spinal cord compression and the need for radiotherapy to bone (Coleman 2007). Approximately 60% of breast cancer patients with bone metastases will experience a pathological fracture with a median time to first SRE of 15 months (Hortobagyi, Theriault *et al*. 1998), therefore patients will be living long enough to not only experience the morbidity associated with SREs but also potentially develop subsequent further SREs. SREs are associated with an increased mortality and therefore therapeutic intervention is essential (Coleman 2006). The prevention of bone metastasis has therefore been a focus of research over the past decade, however, in early breast cancer one of the major challenges is identifying which patients are likely to develop bone metastases and require tailored anti-cancer therapy.

Disseminated tumour cells are detectable in the bone marrow of a third of patients with early breast cancer without any clinical manifestations of bone metastasis (Braun, Vogl *et al*. 2005). Presence of DTCs in the bone marrow correlates with a poorer prognosis for both disease free survival and overall survival, and 50% of patients with detectable DTCs will relapse during 10 years post diagnosis (Braun, Vogl *et al*. 2005). The persistence of DTCs can occur in ~15% of patient after completion of primary therapy and are an independent adverse prognostic factor for both disease free survival and overall survival at 5 years (Janni, Vogl *et al*. 2011). The additional challenge is that these DTCs may be



Adapted from Holen I (2012). Pathophysiology of bone metastases. In Handbook of cancer related bone disease. Ed Coleman RE, Abrahamson PA, Hadji P, 2nd Edition. Bioscientifica, Bristol P39-59
Multiphoton image kindly provided by Gloria Alloca

Figure 1.4. Breast cancer cells home to bone and may remain dormant prior to proliferation and formation of metastases.

Tumour cells home to vascular areas within bone where they interact with bone cells and can enter a state of dormancy/quiescence for many years prior to growth. They are in close contact with bone and are thought to occupy the HSC and MSC niches (left). Following unknown triggers, the tumour cells re-gain the ability to proliferate and ultimately form bone metastases or spread to other metastatic sites (right).

in state of dormancy/quiescence and often do not express the proliferation antigen Ki67 (Pantel, Schlimok *et al*. 1993). Moreover, DTCs express high levels of CD44, suggesting a cancer stem cell phenotype (Balic, Lin *et al*. 2006), thus conferring resistance to chemotherapy used in the treatment of early breast cancer.

1.2 Systemic treatment of early breast cancer – the emergence of bisphosphonates.

1.2.1 Historical development of systemic adjuvant therapies.

Systemic chemotherapy was first used in early breast cancer (adjuvant chemotherapy) in the 1960s, however, it was not until the 1970s when it became standard treatment following the first randomised study conducted by Gianni Bonadonna (Bonadonna, Brusamolino *et al*. 1976). The study evaluated the activity of combination chemotherapy with cyclophosphamide, methotrexate and 5-fluorouracil in the adjuvant setting, after surgery for node positive breast cancer, and demonstrated an increased survival with adjuvant chemotherapy. Over the next decades, research increased the number of systemic therapies available for adjuvant breast cancer therapy including new chemotherapy drugs such as taxanes (reviewed in Di Leo (Di Leo, Ciarlo *et al*. 2004)), endocrine therapies like aromatase inhibitors and tamoxifen (Howell, Cuzick *et al*. 2005) and monoclonal antibodies to the HER2 receptor (Romond, Perez *et al*. 2005). All of these therapies directly target proliferating tumour cells, however, with increasing evidence that the bone microenvironment may be a potential sanctuary for disseminated tumour cells that may be resistant to conventional systemic anti-cancer, disruption of the interactions between tumour cells and the bone stromal cells became an attractive prospect.

The first large adjuvant breast cancer clinical trials evaluating the addition of bone targeted therapy with drugs called bisphosphonates began in the 1990s. Three large trials assessed the use of the bisphosphonate clodronate, for 2-3 years, in addition to standard adjuvant therapy. The results were conflicting with two trials reporting an improved overall survival with clodronate (Powles, Paterson *et al*. 2006; Diel, Jaschke *et al*. 2008) but the third reporting an increase in extraosseous metastases (Saarto, Vehmanen *et al.* 2004) (Fig 1.5). As a result of these inconclusive outcomes, further clinical trials opened

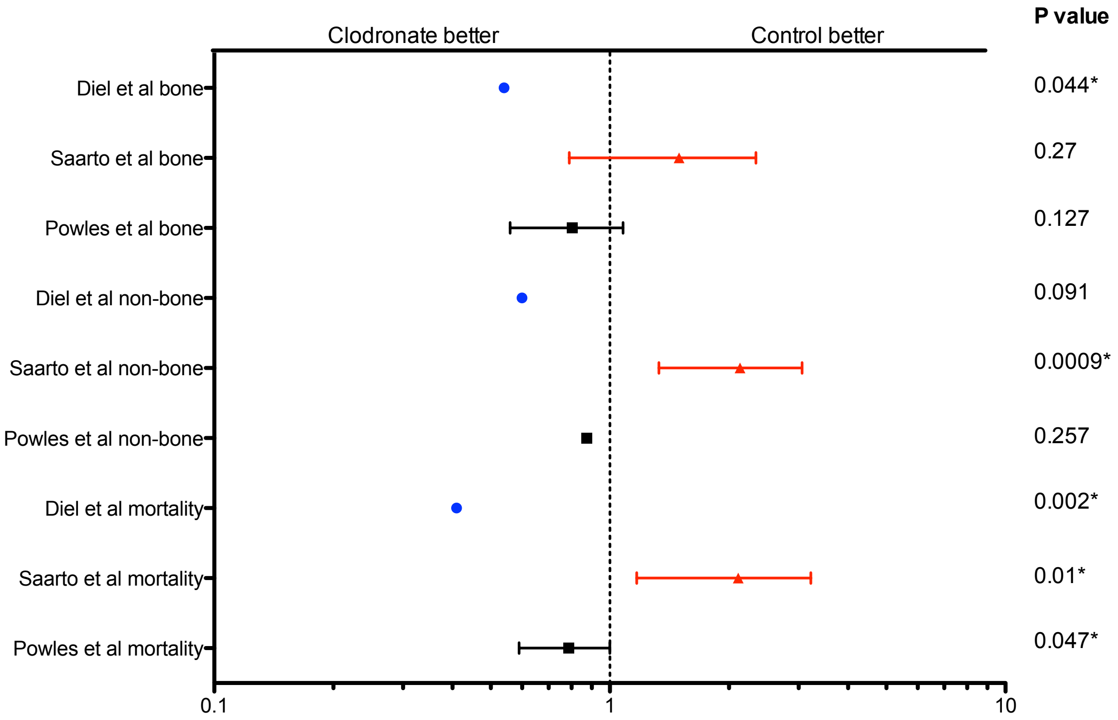


Figure 1.5 Recurrence events and mortality from adjuvant clodronate trials in early breast cancer.

Three pivotal trials conducted by Diel (Diel, Jaschke *et al.* 2008), Saarto (Saarto, Vehmanen *et al.* 2004) and Powles (Powles, Paterson *et al.* 2006) evaluated addition of clodronate to standard adjuvant therapy and showed conflicting results. Data points represents ratio of events at 5 years with 95% CI if quoted in original paper from these 3 trials. Events are grouped into bone, non-bone and mortality. * = Significant p value.

recruiting larger numbers of patients to clodronate (NSABP-B-34), or utilising the newer more potent bisphosphonate zoledronic acid in addition to standard adjuvant therapies (AZURE, SUCCESS, NATAN, AZAC) (summarized in Wilson *et al* (Wilson and Coleman 2011)).

1.2.2 Mechanism of action of zoledronic acid

Bisphosphonates have been extensively utilised in pathological bone disease including osteoporosis and Pagets disease after they were first discovered in the 1960s to prevent pathological calcification (Francis, Russell *et al*. 1969). Bisphosphonates have a pyrophosphate-carbon-pyrophosphate backbone with two additional side groups attached to the carbon atom. The first side group is commonly a hydroxyl group which has high affinity for calcium, but the variations in the second side group have separated the bisphosphonates into those that contain a nitrogen side arm i.e zoledronic acid and those that do not i.e clodronate (Rogers, Gordon *et al*. 2000) (Fig 1.6). Zoledronic acid has a potency in humans that is around 40 fold greater than that of clodronate (Russell, Watts *et al*. 2008) and binds avidly to the bone surface, with a half life in serum of ~2 hours, with drug rapidly bound to bone or cleared by the kidneys (Lin 1996). The drug then remains bound to bone and is taken up by osteoclasts during the resorption process. The effect of zoledronic acid on osteoclasts has been well documented, inducing apoptosis in these cells both *in vitro* and *in vivo* (Hughes, Wright *et al*. 1995), by inhibition of key enzymes within the mevalonate pathway. The mevalonate pathway is responsible for cholesterol synthesis in mammals and is also responsible for post-translational modification of proteins including prenylation of small GTPases such as Rho, Ras and Rac (Luckman, Hughes *et al*. 1998). Zoledronic acid inhibits farnesyl pyrophosphate (FPP) synthase (van Beek, Pieterman *et al*. 1999) (Fig 1.6), preventing prenylation of small GTPases, which are responsible for many cellular functions in osteoclasts including apoptosis and cell membrane integrity (Coxon and Rogers 2003). This negative effect on bone resorbing osteoclasts explains the effective clinical use of bisphosphonates in osteoporosis, and this clinical application was extended to the oncology setting with subsequent evidence that zoledronic acid could prevent bone loss associated with established bone metastases and bone loss associated with anti-cancer therapy (reviewed by Coleman *et al* (Coleman, Rathbone *et al*. 2013) (Coleman 2004)).

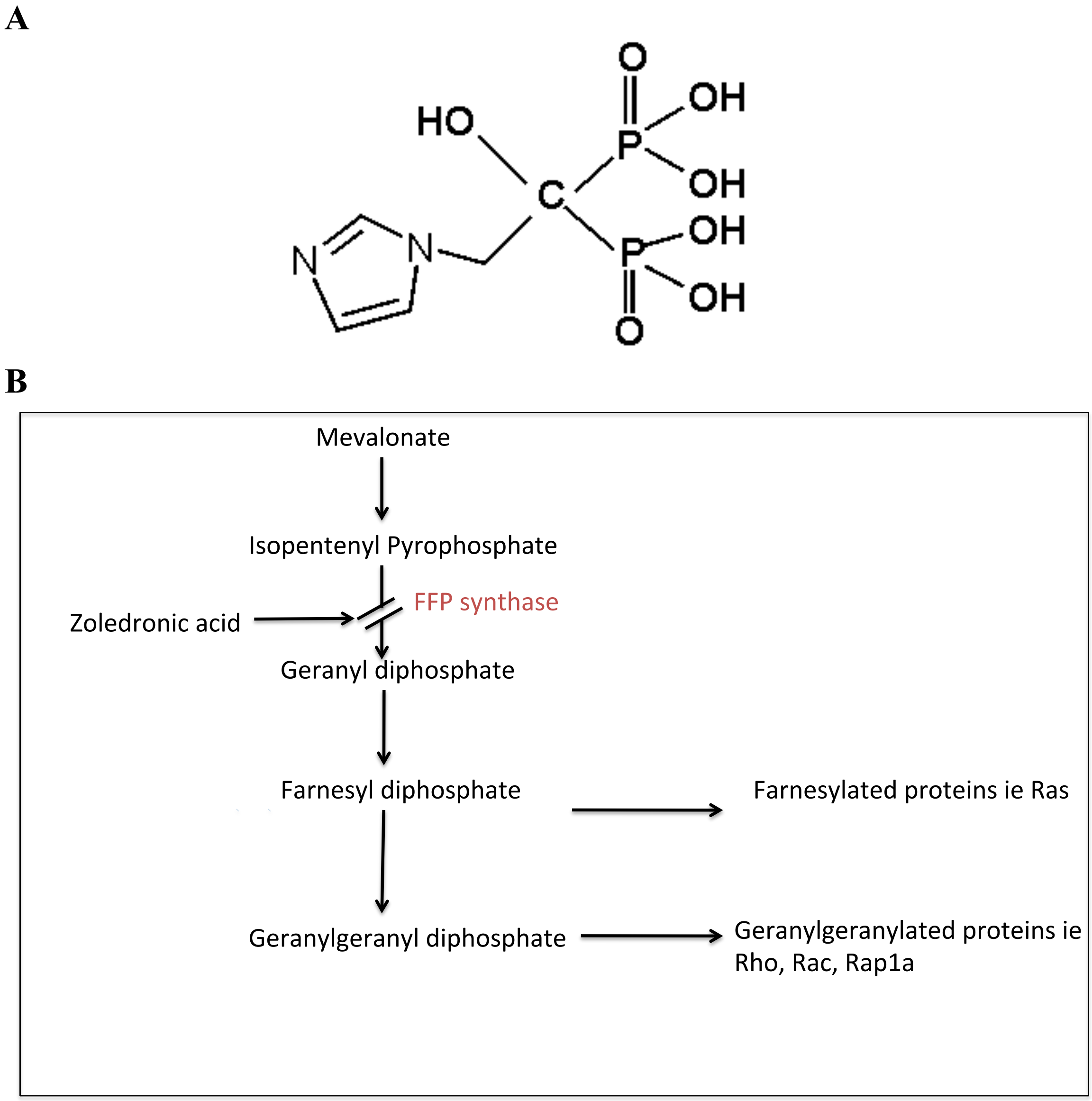


Figure 1.6 Structure and effect of zoledronic acid on the mevalonate pathway.

Zoledronic acid is a nitrogen containing bisphosphonate (A) and inhibits FFP synthase preventing downstream prenylation of small GTPases including Rho, Ras and Rac (B).

What has been of great interest is the potential ability of zoledronic acid to have an anti-tumour effect and prevent the formation of bone metastases in the adjuvant setting. There is a wealth of *in vitro* and *in vivo* evidence to suggest both an indirect anti-cancer effect of zoledronic acid by modification of the bone microenvironment, but also a direct anti-tumour effect in breast cancer.

1.2.3 Zoledronic acid modifies the bone microenvironment – implications for tumour survival.

There is evidence that zoledronic acid can directly affect many cells found within the bone microenvironment. *In vitro*, zoledronic acid (20μM) has been shown to reduce the ability of MSC to proliferate after 48 hours of treatment, with a corresponding 45% decreased ability to migrate through fibronectin coated membranes in response to serum. In addition, MCSs treated with zoledronic acid had an impaired ability to attract ER+ve MCF7 breast cancer cells due to a decreased secretion of chemo-attractants such as RANTES and IL-6 (Gallo, De Luca *et al*. 2012). *In vivo,* zoledronic acid showed a trend to increasing HSC number in male C57BL/6 mice aged 4- and 16-weeks treated with 200μg/kg of zoledronic acid weekly for 4 weeks, which was due to an increase in the osteoblastic numbers rather than a reduction in HSC mobilization or an alteration in the bone vasculature (Soki, Li *et al*. 2013). Zoledronic acid has well documented cytotoxic effects on osteoclasts, as previously discussed, but has also been reported to effect osteoblasts. Studies report conflicting results on the effects of zoledronic acid on osteoblasts with *in vitro* evidence that zoledronic acid (5μM) reduced viability of MG63, a human osteoblast cell line, and prevented normal functioning of these cells as evidenced by a reduction in gene expression of alkaline phosphatase and osteocalcin (Basso, Silveira Turrioni *et al*. 2013). This inhibitory effect of zoledronic acid on osteoblast viability has been confirmed in other studies and can prevent breast cancer cells from migrating in response to osteoblast-secreted factors (Kaiser, Teufel *et al*. 2013). In contrast, human osteoblast differentiation appeared to be enhanced by zoledronic acid (Koch, Merkel *et al*. 2011). The difference in effect in these studies may be due to different doses of zoledronic acid. Corrado *et al* showed that zoledronic acid doses <0.1μM enhance human osteoblast proliferation, but at concentrations greater than this, there was an inhibition of cell proliferation and apoptosis of human osteoblasts (Corrado, Neve *et al*.

2010). Alterations in osteoblast numbers may affect tumour growth since these cells contribute to the HSC and MSC niche as previously discussed.

Zoledronic acid has also been reported to affect other cells that are present within the bone microenvironment, including macrophages and endothelial cells. Zoledronic acid inhibits proliferation of endothelial cells *in vitro* (Wood, Bonjean *et al.* 2002), although this has not been demonstrated *in vivo*. Macrophages are able to take up bisphosphonates due to their phagocytic properties and, *in vitro,* change from a pro-tumour phenotype to an anti-tumour phenotype upon treatment with zoledronic acid (Tsagozis, Eriksson *et al.* 2008; Veltman, Lambers *et al.* 2010), with supportive evidence that tumour associated macrophages absorb (Rogers, Wind *et al.* 2013), and are attenuated by zoledronic acid *in vivo* (Melani, Sangaletti *et al.* 2007; Coscia, Quaglino *et al.* 2010).

The implication of this effect of zoledronic acid for tumour growth in the bone microenvironment is evidenced from *in vivo* animal studies and clinical studies evaluating the effect on disseminated tumour cells in bone marrow. In animal models, the evaluation of the effect of zoledronic acid using preventative treatment protocols (treatment initiated before or on the day of breast tumour cell injection), models the effect of the drug in early breast cancer, in the absence of bone metastasis. Administration of preventative zoledronic acid daily (3μg/kg) or weekly (20μg/kg) commenced 24 hours prior to tail vein injection with a bone homing subclone of MDA-MB-231 oestrogen negative (ER-ve) breast cancer cells to female balb/c nude mice significantly reduced skeletal tumour burden (Daubine, Le Gall *et al.* 2007). Similar effects were found with another bisphosphonate, ibandronate (10μg/kg/day); when used on a preventative protocol in male athymic rats injected with MDA-MB-231 cells, ibandronate decreased the number of metastases (Neudert, Fischer *et al.* 2003). In a similar experiment using olpadronate 1.6μmol/kg/day starting 2 days before the intracardiac injection of MDA-MB-231 cells, the formation of bone metastases was significantly less compared to untreated controls (van der Pluijm, Que *et al.* 2005). Some studies have shown an increase in tumours sitting outside, but in close contact to bone, in association with a decrease in skeletal metastases with the administration of bisphosphonates; female balb/c nude mice treated with risedronate 4μg/day starting 7 days prior to intracardiac injection of MDA-MB-231 cells showed a significant reduction in the number of bone metastases, however, there was an associated increase in tumour burden located outside bone (Sasaki, Boyce *et al*. 1995). A similar effect to this was noted using the same preventative model but an alternative bisphosphonate YH529 (Sasaki, Kitamura *et al*. 1998). These data show that use of bisphosphonates in 'early' breast cancer, before metastases have formed, can reduce the number of bone metastases but they could potentially cause the tumour cells to move to sites outside of bone.

In clinical studies both zoledronic acid and ibandronate are reported to decrease the number of DTCs in bone marrow aspirates from breast cancer patients (Table 1.2) (Aft, Naughton *et al*. 2010; Rack, Juckstock *et al*. 2010; Hoffmann, Aktas *et al*. 2011; Banys, Solomayer *et al*. 2013). Since DTCs have not developed autonomous growth, it is likely that this effect is mediated through bisphosphonate-induced changes in the bone microenvironment, rather than a direct anti-tumour effect. Further evidence of the indirect anti-tumour effect of zoledronic acid, via modification of the bone microenvironment, has been shown in large adjuvant breast cancer clinical trials as discussed in section 1.3.

1.2.4 Zoledronic acid has direct anti-tumour effects in breast cancer

The intrinsic properties of tumour cells that ensure their survival is the ability to proliferate, limit apoptosis, invade and adhere to stroma and ensure an adequate blood supply by stimulating neoangiogenesis. Zoledronic acid has been shown to adversely affect all of these intrinsic tumour properties in breast cancer cell lines. Zoledronic acid (50μM) can inhibit proliferation and induce apoptotic cell death in both ER+ve and ER-ve breast cancer cell lines (Woodward, Neville-Webbe *et al*. 2005; Rachner, Singh *et al*. 2010; Almubarak, Jones *et al*. 2011; Ibrahim, Mercatali *et al*. 2012) (Fromigue, Lagneaux *et al*. 2000; Senaratne, Pirianov *et al*. 2000). The doses of zoledronic acid used in these studies are often higher than those that would be expected to be attainable *in vivo* (>10μM) or in the clinical setting (>1-2μM). Epithelial to mesenchymal transition, a process necessary for invasion of tumour cells, was inhibited in triple negative breast cancer cells in response to treatment with zoledronic acid (Schech, Kazi *et al*. 2013) and invasion of MDA-MB-231 cells can be inhibited by zoledronic acid at low doses (<1μM) (Boissier, Ferreras *et al*. 2000). Inhibition of invasion by zoledronic acid at low doses is thought to be due to effects on the mevalonate pathway, compared to higher concentrations, which may be acting via inhibition of matrix metalloproteinases

Table 1.2 Summary of breast cancer clinical trials evaluating the effect of bisphosphonates on disseminated tumour cells (DTCs) in bone marrow aspirates.

Author	Patient population	Treatment protocol	Outcome measure	Results	Comments
Banys et al (2013)	N= 96. DTC+ve marrow at baseline. Majority T1N0 2/3 postmenopausal 25% ER+ve, HER2-ve	Standard adjuvant therapy +/- ZOL Q4/52 for 24 months	Primary; Change in DTC number at 12 Secondary; change in DTC number at 24 months, safety, bone mets free survival, DFS.	DTC count 12 months; control 27%, ZOL 12%. DTC count 24 months; control 16%, ZOL 0%*	Patients with persistence of DTCs at 12 months post diagnosis had a shorter OS* (no change in DFS)
Aft et al (2010)	N=120 Any DTC status at baseline All patients >T2N1 50% postmenopausal 56% ER-ve	Neo-adjuvant epirubicin ($75mg/m^2$)+docetaxel ($75mg/m^2$) x4 + adjuvant epirubicin x2 +/- 4mg ZOL Q3/52 for 12 months	Primary; Change in DTCs at 3 months Secondary; changes in NTx, osteocalcin, BSAP, BMD baseline, 3, and 12 months	DTC count at 3 months; control 47%, ZOL 30%.	DTCs detectable at baseline in 43% ZOL, 48% control. In patients who were DTC-ve at baseline, appearance of DTCs at 3 months occurred in 40% control, 13% ZOL* No significant effect of ZOL on DFS or OS
Rack et al (2008)	N=31 DTC+ve >6 months since diagnosis	Monotherapy with ZOL Q4/52 for 6 months	Primary; Change in DTCs at 8 months	DTCs declined to persist in 13%	
Hoffmann et al (2011)	N=54 DTC+ve marrow 2-10 years post diagnosis	Monotherapy with PO ibandronte (50mg/day) for 6 months	Primary;Change in DTC at 6 months	DTCs found in 18/54 patients. After 6 months ibandronate 3/17 remained DTC +ve	Patients with DTC+ve marrow after 6 months ibandronate continued therapy for a further 6 months with complete eradication of DTCs

Key; ER=oestrogen receptor, Q4/52= every 4 weeks, ZOL=zoledronic acid, OS=overall survival, DFS= disease free survival, BSAP= bone specific alkaline phosphatase, BMD= bone mineral density, PO= oral administration, *statistically significant p value <0.05.

(Clezardin, Ebetino *et al*. 2005). Zoledronic acid has also been shown to reduce endothelial cell proliferation and angiogenesis *in vitro* (Wood, Bonjean *et al*. 2002). The direct anti-tumour effect of zoledronic acid has also been shown in *in vivo* models of tumours implanted outside bone. 4T1 breast cancer cells spontaneously metastasise to viscera and treatment of mice implanted with 4T1 cells with zoledronic acid IV 5µg every fourth day from day 7-19 resulted in significantly less visceral metastases in lung and liver (Hiraga, Williams *et al*. 2004). These data are in contrast to data published by Ottewell *et al* who showed, in a murine model of sub-cutaneous MDA-MB-436 tumours; treatment with zoledronic acid 100µg/kg weekly for 6 weeks did not alter tumour size compared to saline control (Ottewell, Monkkonen *et al*. 2008). These contrasting results may reflect the differing sensitivities of breast cancer cell lines to zoledronic acid and the different dosing regimes of the drug.

The direct anti-tumour effects of zoledronic acid in clinical studies can be gained from the neo-adjuvant setting, when serial tumour biopsies can show cellular effects, often in combination with chemotherapy. There is both *in vitro* and *in vivo* data showing a synergistic relationship between zoledronic acid and chemotherapy. Neville-Webbe *et al* showed that treatment of MCF7 cells with 0.05µM doxorubicin followed 24 hours later by 25µM zoledronic acid for 1 hour increased apoptosis compared to control, either drug alone or zoledronic acid followed by doxorubicin (Neville-Webbe, Rostami-Hodjegan *et al*. 2005). Other cell lines including MCF7 and MDA-MB-436 have shown synergy with zoledronic acid and paclitaxel (Jagdev, Coleman *et al*. 2001; Neville-Webbe, Evans *et al*. 2006). These results have been confirmed *in vivo*, with the combination of weekly doxorubicin (2mg/kg) followed 24 hours later by zoledronic acid (100µg/kg) causing maximal inhibition of growth in sub-cutaneous tumours in mice inoculated with MDA-MB-436 cells and treated for 6 weeks (Ottewell, Monkkonen *et al*. 2008). The results of neo-adjuvant clinical trials showing a direct anti-tumour effect of zoledronic acid are discussed further in section 1.3.

1.3 Adjuvant and neoadjuvant breast cancer clinical trials of zoledronic acid in addition to standard therapy.

1.3.1 Adjuvant Zoledronic acid clinical trials – modification of the bone microenvironment is influenced by menopausal status.

Several adjuvant clinical trials have evaluated the addition of zoledronic acid to standard therapy for breast cancer patients. The largest of these trials include AZURE (n=3340), ABCSG-12 (n=1803) and ZO-FAST (n=1065) (Fig 1.7). AZURE recruited patients with Stage II/III breast cancer. The population was a mixed menopausal status; premenopausal (45%), unknown menopausal status (9.7%), <5 years since menopause (14.7) and >5 years since menopause (31%), and included both ER+ve patients (78.9%) and ER-ve patients (21%). Patients were randomised to receive standard adjuvant therapy +/- zoledronic acid at decreasing frequency of dosing for 5 years. The primary endpoint was disease free survival (DFS) with secondary endpoint of overall survival (OS), and the results of the 59-month follow up were published in 2011 (Coleman, Marshall *et al*. 2011). There was no significant difference between the control and zoledronic acid groups for DFS or OS, However, the results of a pre-planned subgroup analysis showed a heterogeneity of treatment effect according to menopausal status on DFS. Patients who were >5 years postmenopausal showed a significantly improved DFS with the addition of zoledronic acid (ZOL) to standard therapy (ST) (DFS; ST 71%, ST+ZOL78.2%, HR 0.75, 95%CI 0.59-0.96 p=0.02), but this effect was not seen in any other patient groups (DFS; ST 77.2%, ST+ZOL 74.1%, HR 1.15, 95%CI 0.97 to 1.36 p=0.11). Patients who were >5 years post menopause also had a significant improvement in OS (ST 78.7%, ST+ZOL 84.6%, HR 0.74, 95%CI 0.55 to 0.98 p=0.04). These data were driven by extraskeletal disease recurrence, being decreased in patients >5 years since menopause but increased in all other patients. A recent update of the AZURE results following 966 DFS events and a median follow up of 84 months, has now demonstrated that there is a significant reduction in bone metastasis in the overall population with addition of zoledronic acid (HR 0.81, 95% CI 0.68-0.97 p=0.022) that is not influenced by menopausal status, but the effects of zoledronic acid on extra skeletal distant recurrence continued to show a decrease in women >5 years postmenopausal but an increase in all other patients (Fig 1.8)(R. Coleman, R. Burkinshaw *et al*. 2013).

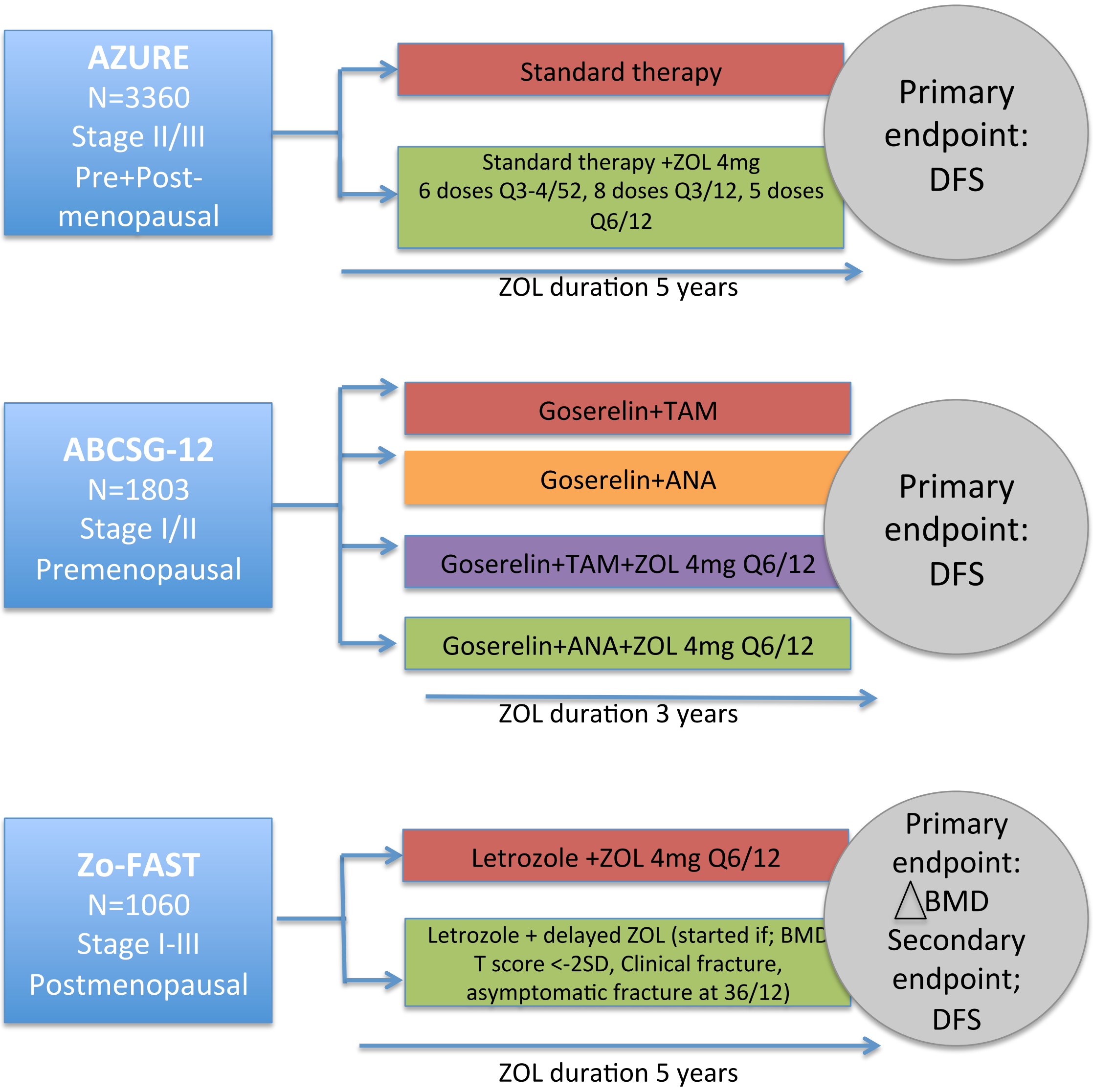
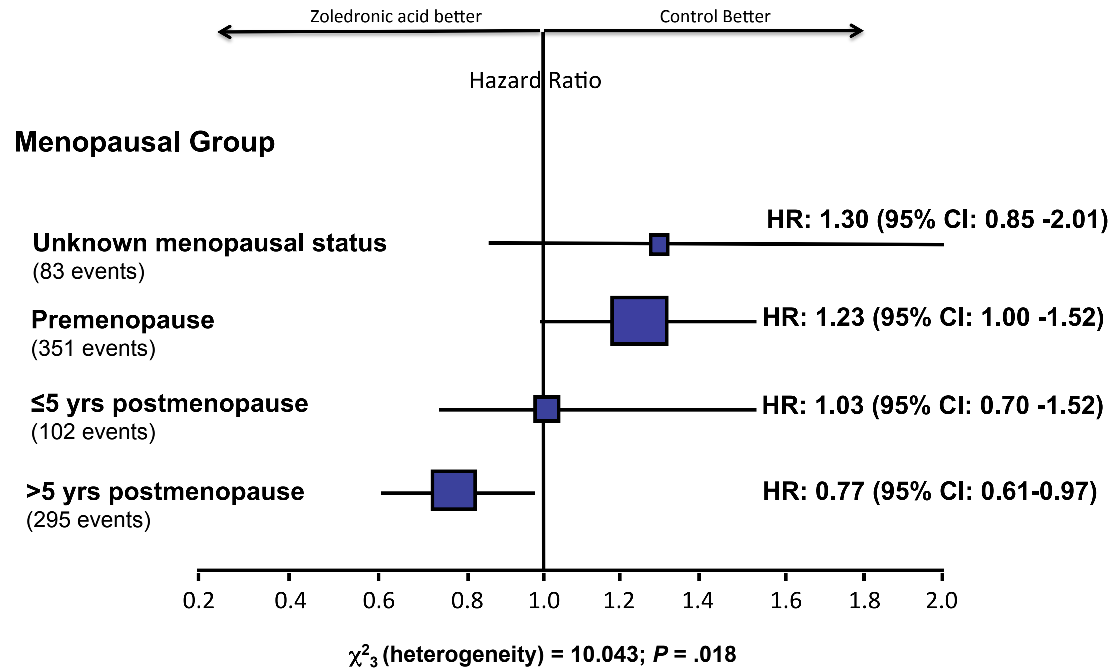


Figure 1.7 Trial schematics of the largest adjuvant clinical trials of zoledronic acid.

Key; ZOL=zoledronic acid, Q3-4/52= every 3-4 weeks, Q3 or 6/12 = every 3 or 6 months, TAM = tamoxifen 20mg daily, ANA = anastrazole 1mg daily, SD= standard deviation, DFS= disease free survival, Δ = change



Adjusted for imbalances in ER, lymph node status, T stage and neo-adjuvant therapy.

Figure reproduced from Coleman RE, Hinsley S, Dodwell D *et al*. Cancer and Bone Society conference, 2013, Miami, Florida; abst LBA001

Figure 1.8 Extraskeletal distant recurrences according to menopausal status at diagnosis from the AZURE trial.

Women who had experience menopause >5 years prior to initiation of zoledronic acid had a HR of 0.77 indicating that the use of zoledronic acid in this population was reducing extraskeletal recurrences. All other menopausal groups had a HR of >=1.0 suggesting no effect or an increase in extraskeletal recurrences.

These data were supported by the results of the ABCSG-12 trial. The population recruited to this trial differed to that of AZURE I that they were all premenopausal women with oestrogen receptor positive disease, but all patients in ABSCG-12 were treated with goserelin, which induced a chemical menopause prior to being randomised to receive tamoxifen/anastrazole +/- zoledronic acid every 6 months for 3 years. The primary endpoints were DFS and secondary endpoints OS and bone mineral density (BMD) assessment. The results of 62-month follow up were published in 2011, focusing on DFS and OS outcomes (Gnant, Mlineritsch *et al.* 2011). Zoledronic acid significantly improved DFS compared to endocrine therapy alone (92% vs 88% p=0.008). This improvement in DFS was for recurrences both in bone and outside of bone. A pre-planned subgroup analysis according to age a study entry demonstrated a significant beneficial effect of zoledronic acid on DFS in women >40 years (HR 0.58, 95% CI 0.4-0.83), and these effects were not seen in women <40 years (HR 0.94, 95%CI 0.57-1.56) (Fig 1.9). Overall survival did not alter with the addition of zoledronic acid.

The data from ABCSG-12 and AZURE have been further supported by ZO-FAST, which reported the 60-month follow up analyses in 2013 (Coleman, de Boer *et al.* 2013). ZO-FAST evaluated addition of zoledronic acid 4mg every 6 months to the aromatase inhibitor letrozole 2.5mg/day for 5 years. The patients were all postmenopausal with oestrogen positive disease, and initiated zoledronic acid at the start of the letrozole (early) or delayed the start of zoledronic acid until evidence of BMD loss or fracture. The primary endpoint of this study was change in BMD at 12 months but pre-planned secondary analyses included DFS and OS. At 60-month follow up, patients who started zoledronic acid with letrozole had a 34% decrease in DFS events (HR 0.66 95%CI 0.44-0.97 p=0.0375). There were fewer local (0.9% vs 2.3%) and distant recurrences (5.5% vs7.7%) in the early zoledronic acid treatment group vs delayed zoledronic acid. There was no difference in OS between the two treatment groups. Exploratory analyses according to menopausal status at randomisation showed that women >5 years postmenopausal or >60 years has a substantially improved OS with early vs delayed zoledronic acid (HR 0.5; p=0.0224). This effect was not seen in women who were recently postmenopausal due to chemotherapy induced ovarian toxicity, oophorectomy or ovarian suppression (Fig 1.10). Women > 5 years postmenopausal or ages >60 also had the largest increase in lumbar spine BMD with early zoledronic acid compared to recently

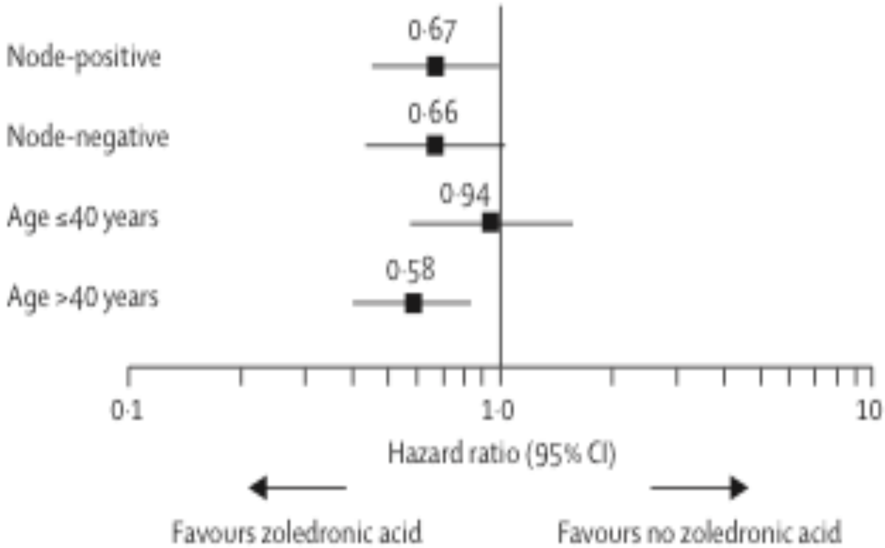


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Figure 1.9 Disease free survival according to nodal status and age at randomization from the ABCSG-12 trial.

Nodal status did not significantly affect the anti-tumour efficacy of zoledronic acid, however, women aged >40 had an improved DFS with zoledronic acid compared to those without. This effect was not seen in women aged<40.

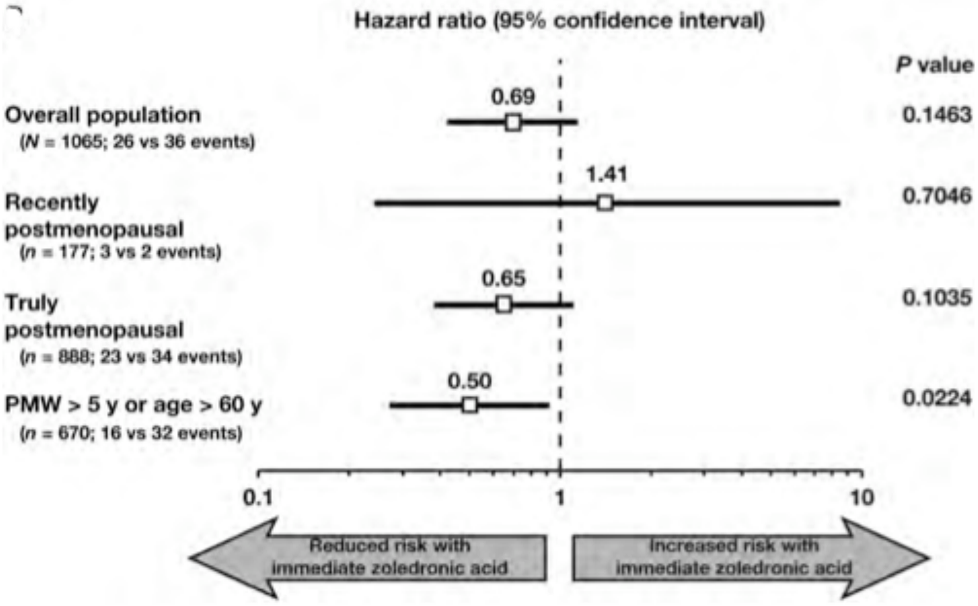

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Figure 1.10. Overall survival by menopausal status at randomization in the ZO-FAST trial.

Women who were >5years postmenopausal or aged >60 had an improved OS with immediate zoledronic acid. This effect was not seen in women who were recently postmenopausal due to chemotherapy induced ovarian failure, oophorectomy or ovarian suppression.

postmenopausal women whose lumbar spine density remained stable with early zoledronic acid. These data show that in women with established menopause, zoledronic acid has more of an effect on bone volume/density in addition to an increase anti-tumour effect compared to recently postmenopausal women.

Together these data show that a subgroup of patients with established menopause and therefore low levels of ovarian hormones may be deriving an increased anti-tumour benefit from adjuvant zoledronic acid. Interestingly, the differential anti-tumour effect of zoledronic acid according to menopausal status is not a drug specific phenomenon, as 2 adjuvant clodronate studies have also shown improved outcomes in postmenopausal women. Powles *et al* treated 1069 Stage I-II, pre- and postmenopausal breast cancer patients with adjuvant clodronate 1600 mg daily for 2 years, and in a subgroup analysis they showed that both the 2 year bone metastasis free survival and OS were significantly improved in postmenopausal women receiving clodronate compared to those receiving placebo. This effect was not seen in premenopausal patients (Powles, Paterson *et al.* 2006). In another adjuvant clodronate trial, NSABP-B34, 3323 patients with Stage I/III breast cancer were randomised to receive clodronate 1600mg daily for 3 years or placebo, with the primary endpoint being DFS. With a median follow up of 90 months, DFS was similar between both groups, however, women aged >50 showed a significantly reduced bone (HR 0.64, 95%CI 0.4-0.95 p=0.047) and non-bone (HR 0.63, 95% CI 0.43-0.91 p=0.014) metastasis free survival (Paterson, Anderson *et al.* 2012).

A meta-analysis of DFS data from 8735 postmenopausal women recruited to 7 different adjuvant bisphosphonate trials with zoledronic acid, clodronate and ibandronate, showed that there was a significant DFS benefit for zoledronic acid with a DFS reduction of 24% (p=0.0006), and this lessened to 18% when the clodronate and ibandronate studies were included, but the DFS improvement with bisphosphonates remained significant (p=0.0075)(Gregory W 2012). These data support the notion that the anti-tumour effect of adjuvant bisphosphonates is likely to be via their modification of the bone microenvironment, rather than a specific pharmacological property of the drugs, since both non-nitrogen and nitrogen-containing bisphosphonates have similar effects.

1.3.2 Neoadjuvant zoledronic acid clinical trials – evidence for a direct anti-tumour effect.

Data on the anti-tumour effects of zoledronic acid has been derived from both prospective neo-adjuvant clinical trials and retrospective analysis from other datasets.

A retrospective analysis of 205 patients who received neoadjuvant chemotherapy (CT)+/- zoeldronic acid (ZOL) in the AZURE trial reported evidence of a direct anti-tumour effect of zoledronic acid. The primary end point of this analysis was residual invasive tumour size (RITS) and secondary endpoints included number of positive lymph nodes. Multivariate analysis of mean RITS showed a significant difference between the two treatment groups with a mean RITS of 27.4 in the CT alone groups vs 15.5 CT+ZOL with a mean difference of 12mm (95%CI 3.5-20.4 p=0.0059). Other factors that were significantly associated with a lower RITS were treatment duration. There was also a trend towards a lower RITS in patients with ER-ve tumours, although this did not reach statistical significance (p=0.0609). No significant difference in positive axillary lymph nodes was found according to treatment group (Coleman, Winter *et al*. 2010).

An exploratory biomarker study of the effects of zoledronic acid 4mg IV given 24 hours after the first cycle of neoadjuvant FEC_{100} chemotherapy (ANZAC study) was recently reported (Winter, Wilson *et al*. 2013). 40 patients with invasive primary breast cancer *in situ* were included in the study and groups were matched for tumour stage, ER- and Her2-receptor status and menopausal status. Primary tumour biopsies were taken at baseline, day 5 and day 21, with a primary outcome measure of change in apoptotic index in the primary tumour between baseline and day 5. Secondary tumour endpoints included changes in proliferation measured by Ki67, growth index (Ki67/apoptosis) and changes in serum vascular endothelial growth factor (VEGF). There was no significant difference in apoptotic index or Ki67 baseline to day 5 between treatment groups. However, there was a significant increase in Ki67 at day 21 compared to baseline in the patients receiving zoledronic in addition to chemotherapy (+184%), an effect not seen in those receiving chemotherapy alone (-26.9%). Serum VEGF was significantly lower at day 5 compared to baseline in patients receiving zoledronic acid+chemotherapy compared to chemotherapy alone (median change pg/ml; -16.8 vs -73.7 p=0.035). The authors conclude that in this biomarker study there is no definite evidence for a direct anti-tumour effect of zoledronic acid, which may be due to timing of sample collection and the use of only a single treatment with zoledronic acid.

A phase II trial evaluated the efficacy and safety of addition of 4mg zoledronic acid every 3 weeks for 12 months commencing with first cycle of neo-adjuvant chemotherapy in 120 women with invasive breast cancer. The primary endpoint included pathological complete response (pCR) in primary tumour. There was no significant difference in pCR rates in the overall population according to treatment received, however, patients with ER-ve tumours showed a trend to higher pCR rates than ER+ve patients (ER-ve pCR rates; ZOL 28%, no ZOL 10%) (Aft, Naughton *et al.* 2010). The secondary endpoints were DFS and OS and were recently reported following 62-month follow up data (Aft, Naughton *et al.* 2012). No significant difference was detected in either secondary endpoint between treatment groups; however, they reported significant differences in the response to zoledronic acid according to ER status, with ER –ve patients showing a significant improvement in DFS and OS with zoledronic acid treatment, an effect not seen in ER+ve patients (Fig 1.11). The lack of benefit from zoledronic acid in the ER+ve patients is in contrast to the results of the adjuvant studies ZO-FAST and ABCSG-12 which recruited only ER+ve patients, but all of those patients were either naturally or chemically postmenopausal whereas the ER+ve population in this trial were young (median age 47) and not receiving ovarian suppressive therapy. The ER-ve population still showed an improved DFS and OS with zoledronic acid, despite not all being postmenopausal, which the authors suggest may indicate that the responsiveness of ER–ve tumour status to zoledronic acid is not dependent upon a postmenopausal status, and may indicate a direct anti-tumour effect of zoledronic acid in this sub population. These data, generated in both adjuvant and neoadjuvant clinical trials, suggest that zoledronic acid may potentially have an enhanced indirect anti-tumour effect via modification of the bone microenvironment in women who are naturally or chemically postmenopausal and could have an enhanced direct anti-tumour effect in ER-ve breast cancer. The molecular mechanisms underpinning this differential effect of zoledronic acid are yet to be determined.

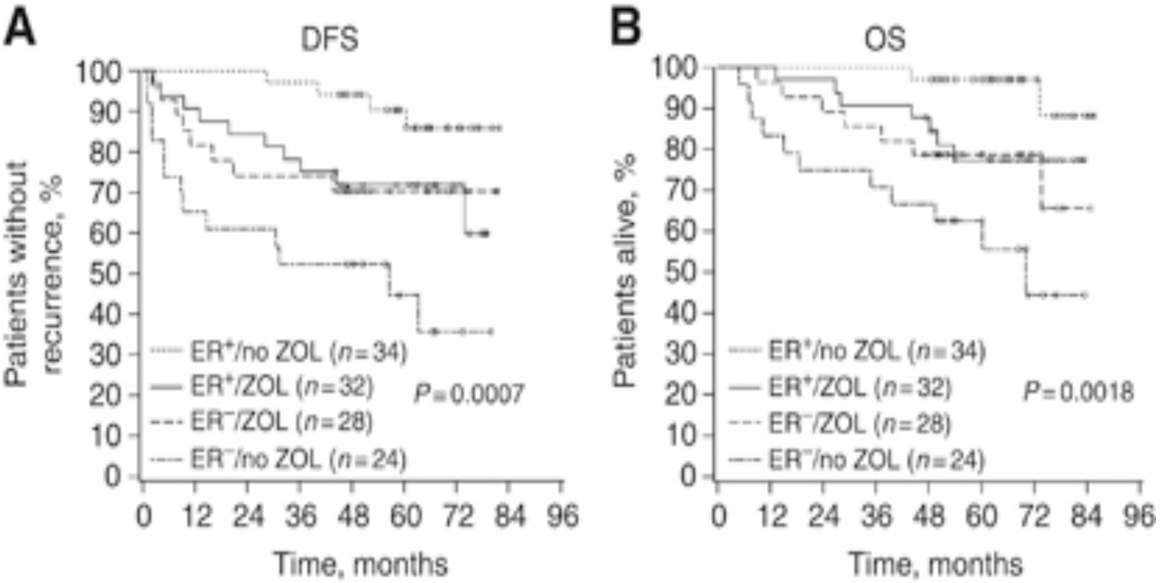

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Figure 1.11 Disease free survival (DFS) and overall survival (OS) according to ER status and treatment received.

Patients with ER-ve tumours had a significant improvement in DFS (A) and OS (B) with the addition of zoledronic acid, an effect not seen in patients with ER+ve tumours.

1.4 Why are menopause and ER status influencing the anti-tumour efficacy of zoledronic acid?

1.4.1 Premenopausal to postmenopausal transition – dynamic changes in both ovarian and pituitary hormones.

The hypothalamic pituitary axis (HPG) is a physiological feedback mechanism whereby the secretion of ovarian hormones (oestrogen, inhibins and progesterone) are controlled by the hypothalamus via pituitary gland secretion of follicle stimulating hormone (FSH) and lutenising hormone (LH), with resulting negative feedback from the ovarian hormones on both pituitary and hypothalamus. Oestogen inhibits release of gonadotrophin releasing hormone (GnRH) and inhibins inhibits FSH (Fig 1.12). Oestrogen is a steroid hormone with different isoforms; oestradiol is the predominant form in premenopausal women and is produced by ovaries, compared to oestrone produced from the adrenal cortex in postmenopausal women (Nelson and Bulun 2001). Inhibins (A and B) are primarily secreted by ovaries during reproductive life (Stenvers and Findlay 2010). The changes in these hormones up to and after the final menstrual period have been well characterized (Hale and Burger 2009), and reproductive aging is associated with a decrease in ovarian secretion of inhibins and oestrogen with a rise in FSH, due to loss of negative feedback. The rise in FSH occurs prior to the final menstrual period (FMP), and can occur up to 3-10 years before menopausal transition (Burger, Hale *et al*. 2007), which has been attributed to a decline in inhibins (Klein, Houmard *et al*. 2004).

At the time of the FMP oestrogen and inhibins fall, and the majority of women will have undetectable levels of inhibins (Burger, Dudley *et al*. 2002), however, the decline in oestradiol is slower and it can remain detectable in serum, with currently available assays, for up to 5 years post FMP (Burger, Dudley *et al*. 1999) (Table 1.3).

The differing levels of endocrine hormones in pre- and postmenopausal women will have differential effects on organs such as bone, due not only to their endocrine effect but also due to differential changes in associated paracrine factors. These paracrine factors may in turn modify both tumour growth and response to therapy.

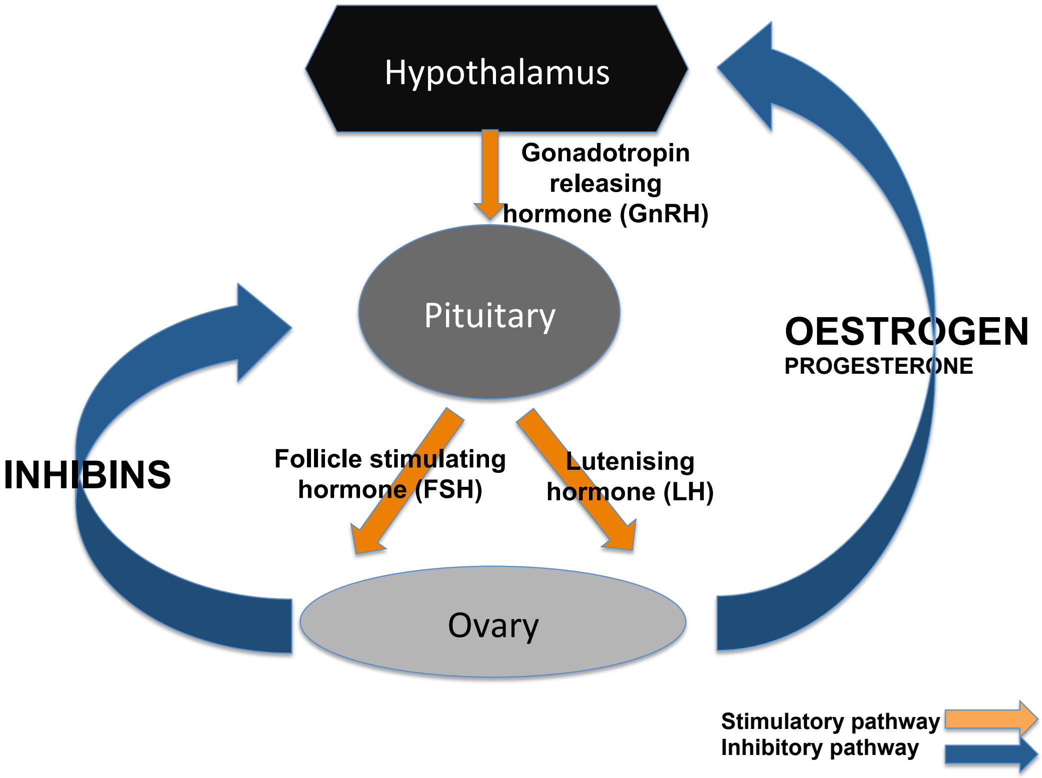


Figure 1.12. The hypothalamic pituitary gonadal axis.

Secretion of the ovarian hormones oestrogen, progesterone and inhibin are stimulated by follicle stimulating hormone and lutenising hormone released from the pituitary under hypothalamic control. The ovarian hormones have negative feedback effects of the pituitary and hypothalamus thus ensuring regulation of this physiological system.

Table 1.3 Changes in endocrine/paracrine agents with alteration in reproductive status. (Serum levels described relative to mid reproductive age).

Typical serum levels are depicted in brackets and are adapted from Burger *et al* (Burger, Dudley *et al*. 2002).

	Mid reproductive age	Late reproductive age	Early menopause transition	Late menopause transition	Established menopause
FSH	Normal (10)	Elevated (20)	Elevated (40)	Elevated (50)	Elevated (100)
LH	Normal	Normal	Elevated	Elevated	Elevated
Oestradiol	Normal (300)	Normal (300)	Normal to elevated (300)	Decreased (150)	Decreased (<50)
Progesterone	Normal	Normal	Normal	Decreased	Decreased
Inhibin B	Normal (50)	Decreased (40)	Decreased (35)	Decreased (30)	Undetectable
Inhibin A	Normal (25)	Normal (25)	Decreased (15)	Decreased (10)	Undetectable

Units of measurement; FSH = iu/l; E2= pmol/l; inhibin A/B=ng/l

1.4.2 Effects of female hormones on bone turnover.

The effects of menopause on bone are well documented, with a loss of bone density postmenopausal, associated with increased fracture risk (Melton, Alothman *et al*. 2003). The cause of this bone loss was for many years, attributed solely to the effects of oestrogen (Manolagas 2000), and supplementation with oestrogen by hormone replacement therapy negated these effects (Riis, Overgaard *et al*. 1995). However, it has been increasingly recognized that bone loss occurs in the perimenopausal phase, when oestradiol levels are still high, and this has been shown to be due to a decline in inhibins and an associated increase in FSH (Riggs, Melton *et al*. 2008).

Inhibins suppress osteoclast and osteoblast activity *in vitro* via inhibition of RANKL (Gaddy-Kurten, Coker *et al*. 2002), suggesting that in the presence of inhibins, bone turnover may be reduced. *In vivo* studies have shown an anabolic effect of continuous administration of inhibin A on the skeleton via an increase in osteoblast activity [Nicks, 2010 #673]. In a cross sectional study of women aged 21-85 (n=188), endocrine hormones were correlated to changes in serum markers of bone formation; bone specific alkaline phosphatase (BSAP), and bone resorption; carboxyterminal telopeptide of type I collagen (CTX). Inhibin A was shown to be the most accurate predictor of changes in bone formation and resorption being negatively correlated with levels of BSAP and CTX (Perrien, Achenbach *et al*. 2006). FSH increases osteoclast differentiation *in vitro* (Zhu, Tourkova *et al*. 2012), and *in vivo* treatment of ovariectomised 14-week old mice with an antibody to β-subumit of FSH, which blocks the biological activity of FSH, prevented OVX-induced bone loss after 4 weeks of treatment. Dynamic histomorphometry showed inhibiting FSH increases all bone formation parameters and inhibits bone resorption parameters (Zhu, Blair *et al*. 2012). Clinical studies have confirmed the link between a high FSH and increased serum markers of bone turnover in large cross sectional studies. Levels of osteocalcin (a marker of bone formation) and N-telopeptide of type I collagen (NTx) (a marker of bone resorption) were correlated with serum changes in female hormones in perimenopausal women (n=2375). High FSH was significantly associated with high NTx and a high osteocalcin, indicating that changes in FSH may be contributing to the changes in bone density at perimenopause (Sowers, Greendale *et al*. 2003). Inhibins were not measured in this study therefore it is not possible to separate the effect of a rising FSH versus a decline in inhibins. In the cross sectional study by Perrien *et al* showing that inhibin A was the most accurate predictor of changes in bone formation and resorption, FSH was also measured. FSH correlated with bone resorption markers

(CTx) but not bone formation markers (BSAP) in perimenopausal women, and did not correlate with any bone turnover markers in pre- or postmenopausal women (Perrien, Achenbach *et al*. 2006). These data therefore suggest a direct stimulatory effect of FSH on osteoclastogenesis *in vitro* and *in vivo,* with additional inhibition of osteoblastogenesis *in vivo*, however, further longitudinal clinical studies evaluating both inhibins and FSH are needed to determine if the inhibitory effects on osteoblastogenesis are mediated through a high FSH or concurrently low inhibins.

These data suggest there will be significant endocrine differences in the bone microenvironment in pre- and postmenopausal women, which may differentially influence the survival and growth of disseminated tumour cells in bone. This may be a direct effect of different hormone levels on bone cell number and activity, as previously discussed, but may also be due to changes in the soluble paracrine growth factors in bone which are under the influence of the female hormones inhibin A and B.

1.4.3 Inhibins interact with members of the TGFβ superfamily of paracrine peptides – implications for breast cancer cell growth.

The ovarian hormones inhibin A and B do not have an identified intracellular downstream signaling pathway, but bring about their effector functions by inhibiting members of the TGFβ superfamily of signaling peptides. These paracrine proteins are abundant in many tissues, including bone, and include activin, TGFβ, bone morphogenetic proteins, anti mullerian hormone and nodal, with effector functions of cell cycle regulation, differentiation and growth (Bierie and Moses 2010). Activin and TGFβ each bind to their respective type II receptors, but both recruit the same type I receptor which results in phosphorylation of the receptor associated Smads 2/3 (Liu and Feng 2010). This interaction between ligand and type II receptor is prevented by inhibins (Vale, Wiater *et al*. 2004). Activin is also bound to a single chain glycosylated peptide, follistatin, which prevents its biological activity and it must be cleaved from follistatin to allow receptor ligand interaction. Activins biological activity is therefore prevented by both inhibins and follistatin (de Kretser, Buzzard *et al*. 2004) (Fig 1.13).

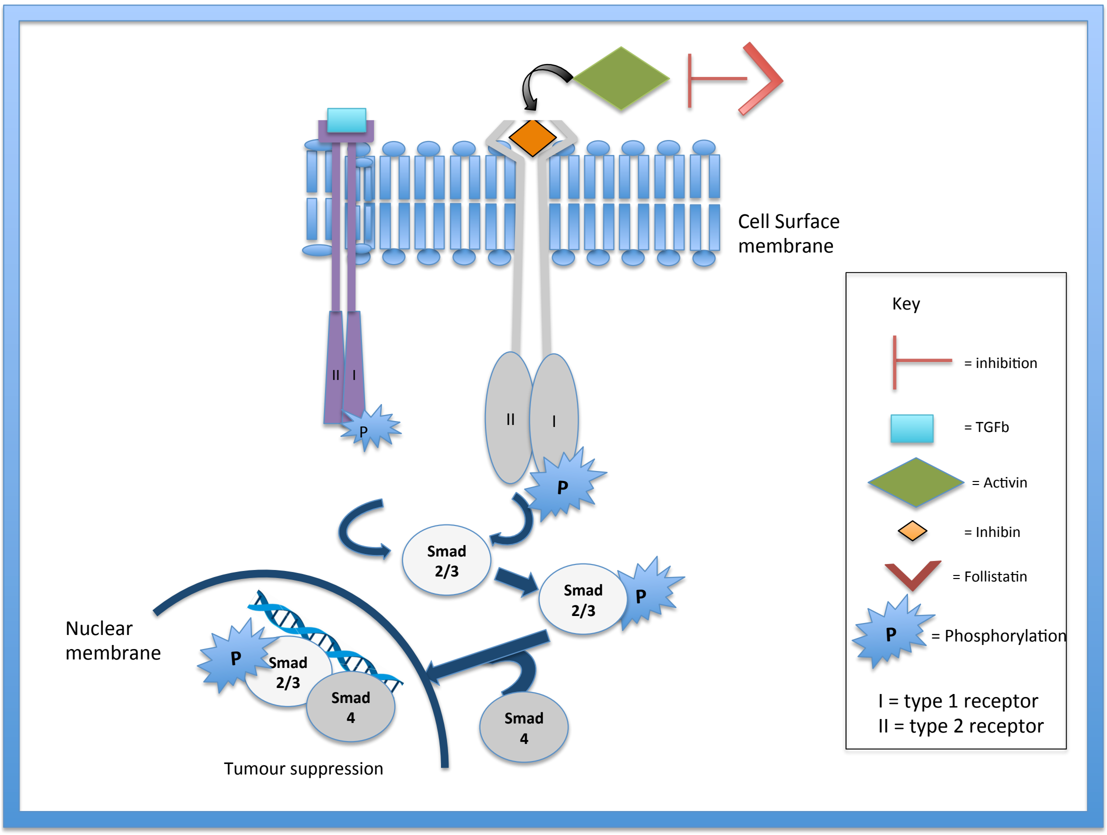


Figure 1.13. Inhibin interacts with the activin type II receptor to bring about its effector functions.

Inhibin binds to the activin type II receptor (ActRII) and prevents activin from binding. In addition to inhibin, activin is also inhibited by follistatin, a single chain glycosylated peptide which binds to activin in the circulation and extracellular spaces and prevents activin from binding to ACRII. In the absence of inhibin and follistatin, activin binds to ActRII and induces phosphorylation of the receptor associated Smads 2/3, these undergo translocation to nucleus to affect DNA transcription. In breast cancers this pathway has a tumour suppressive role.

Paracrine changes in the tumour microenvironment have a direct effect on breast cancer cell growth- influence of ER status.

Activin is produced by both ER+ve and ER-ve breast cancer cells *in vitro*. It acts as a tumour suppressor, and early studies suggested the tumour suppressive action was greatest in ER+ve cells due to loss of activin signaling mechanisms in ER-ve cells (Kalkhoven, Roelen *et al*. 1995). However, these early studies did not evaluate breast cancer cell secretion of the activin inhibitor follistatin. Follistatin has been shown to cause insensitivity to the growth inhibitory effects of exogenous activin in ER-ve cells, which can be reversed by removal of follistatin from culture medium (Razanajaona, Joguet *et al*. 2007). The tumour suppressive effect of activin has been further shown in clinical breast cancer samples, wherean association was shown between loss of activin receptor (ActRII) expression and increasing grade of tumour (Jeruss, Sturgis *et al*. 2003), indicating that tumour cells down regulate their response to the growth inhibition from activin as they become more aggressive. In breast cancer patients without metastases, serum activin levels are raised and these levels fall after breast cancer surgery. However, serum levels of activin do not correlate with poor prognostic factors such as lymph node involvement or tumour grade, indicating activin is secreted as a consequence of malignancy but is not the cause (Reis, Cobellis *et al*. 2002).

TGFβ1 is produced by breast cancer cells *in vitro* at significantly higher levels than benign breast tissue (Travers, Barrett-Lee *et al*. 1988). In contrast to its effect in established bone metastases, in early breast tumour development its role is that of a tumour suppressor as shown in transgenic mouse models in which blockade of the TGFβ receptor II (TGFβRII) increased the number of spontaneous mammary tumours (Yang, Dukhanina *et al*. 2002). TGFβ therefore appears to have both a tumour suppressive action in early tumorigenesis and a tumour promoting action in later stages of tumourigenesis.

The activin/TGFβ receptor associated Smad2 protein has been shown to be a tumour suppressor in clinical breast cancer specimens, with a loss of phosphorylated Smad2 correlating with a decreased disease free survival in women with early stage disease (Xie, Mertens *et al*. 2002).

Paracrine changes in the bone microenvironment can affect breast cancer cell growth.

The role of activin in bone is almost directly opposite to that of inhibins, with *in vitro* data showing that activin enhances osteoclast formation via increasing RANK expression (Sugatani, Alvarez *et al*. 2003). The effect of activin on osteoblast formation *in vitro* is less well defined, with reports that it both stimulates (Gaddy-Kurten, Coker *et al*. 2002) and inhibits (Eijken, Swagemakers *et al*. 2007) osteoblast formation. Inhibiting activin *in vivo* by delivery of a soluble activin receptor fusion protein has similar effects to that of inhibins, resulting in an increased bone volume via an increase in osteoblast number, suggesting that activin inhibits osteoblastogenesis (Chantry, Heath *et al*. 2010).

TGFβ1 has differential effects on osteoblasts and osteoclasts *in vivo* by increasing the former and decreasing the latter (Edwards, Nyman *et al*. 2010). TGFβ1 is also secreted by osteoblasts into the bone matrix and is subsequently liberated upon osteoclastic bone resorption. This release of TGFβ attracts osteoblasts to sites of resorption and TGFβ therefore plays an integral role in the coupling of bone formation to bone resorption (Iqbal, Sun *et al*. 2009).

Effects of activin and TGFβ1 on breast cancer cell growth in bone have been reported in studies using *in vivo* models. Breast cancer cells in bone increase PTHrP secretion in response to TGFβ that promotes osteoclastogenesis (Buijs, Stayrook *et al*. 2011), suggesting a tumour promoting action of TGFβ in established bone metastases. TGFβ also increased expression of CXCR4 on MDA-MB-231 cells which promotes bone homing, adhesion to bone and formation of bone metastases *in vivo*, an effect which is lost if TGFβ is inhibited in the cell line (Dunn, Mohammad *et al*. 2009). Delivery of a soluble activin receptor fusion protein to intact mice, prior to intracardiac inoculation with MDA-MB-231 cells, prevented the development of osteolytic bone lesions indicating that low levels of activin in bone may prevent the formation of bone metastasis (Chantry, Heath *et al*. 2010). These results suggest that modifying the bone environment by inhibiting bone turnover prior to the arrival of tumour cells may prevent bone metastases. Whether this is mediated through administration of activin inhibitors or other compounds such as bisphosphonates, the results are similar. Knockdown of the activin receptor in the same MDA-MB-231 cells prior to intracardiac injection did not affect the formation of bone metastasis compared to parental cell line (Chantry, Heath *et al*. 2010), However, another group showed that knockdown of the activin/TGFβ receptor associated intracellular protein Smad2 in MDA-MB-231 resulted in a more aggressive phenotype with rapid formation of bone metastasis (Petersen, Pardali *et al*. 2010). Breast cancer cells therefore appear to be influenced by the activin/TGFβ pathway, with increased tumour propensity if Smad2 levels are decreased.

Together these data suggest that the paracrine factors, TGFβ, activin and the activin inhibitor follistatin, may have relevant effects on tumour proliferation in early breast cancer. The effects on breast cancer cells in either the local primary tumour environment or the bone microenvironment may alter according to the levels of the female hormone inhibin A and B, which are high in premenopausal women and low in postmenopausal women. It is not known if zoledronic acid can affect these paracrine proteins in either the primary tumour or bone microenvironment, or if the absence or presence of female hormones can interact with the drug to alter activin, follistatin, TGFβ or the ratio between these.

1.5 Summary and Hypothesis

As discussed there is emerging clinical data that bisphosphonates such as zoledronic acid can improve outcomes in early breast cancer in 2 groups of patients; postmenopausal women and patients with ER-ve breast tumours. Zoledronic acid is likely to be routinely incorporated into clinical practice for neo/adjuvant treatment of these patients, and therefore there is a need to understand how reproductive hormones and the ER status of breast cancers interact with the drug to alter its anti-tumour action.

In postmenopausal women the effects of zoledronic acid may be mediated through modification of the bone microenvironment, with subsequent effects on DTCs either by alteration in bone cell activity or number, or availability of the soluble paracrine growth factors TGFβ, activin and the activin inhibitor follistatin. This beneficial effect may dependent upon a postmenopausal endocrine profile with low levels of the ovarian hormones oestradiol and inhibins. In women with ER-ve tumours, the beneficial effect of zoledronic acid may be mediated through direct effects on the soluble paracrine growth factors TGFβ, activin and the activin inhibitor follistatin in the local tumour environment, which are not occurring in ER+ve tumours.

Therefore the hypothesis of this research was; Zoledronic acid can affect TGFβ, activin and the activin inhibitor follistatin in both the primary tumour environment and in bone, but these effects are differentially altered according to presence or absence of female hormones and ER status of primary tumours.

1.6 Aims

Chapter 3; AZURE translational study; To evaluate if biochemical classification of menopausal status using FSH, oestradiol and inhibin A is more reliable than patient reported clinical classification in selecting patients for adjuvant zoledronic acid. To assess the predictive value of a treatment interaction with zoledronic acid for each individual hormone. To assess the prognostic value of each individual hormone for bone and distant breast cancer recurrence.

Chapter 4; ANZAC endocrine sub-study study; To assess if serum levels of TGFβ, activin and follistatin are altered by zoledronic acid in patients being treated with neoadjuvant chemotherapy +/- zoledronic acid. To evaluate if alterations in the serum levels of the paracrine proteins are affected by menopausal status or ER status at baseline.

Chapter 5; To evaluate if ER+ve and ER-ve breast cancer cells secrete TGFβ, activin or follistatin *in vitro* and the effects of these proteins on proliferation. To assess if zoledronic acid differentially affects these paracrine factors according to ER status of cancer cells *in vitro* and *in vivo*.

Chapter 6**;** To evaluate the effects of inhibin A on bone volume, bone cell number and bone levels of activin and follistatin *in vivo.* To assess the effects of zoledronic acid on bone cell number and bone levels of activin and follistatin *in vivo.*

Chapter 2. Materials and Methods.

2.1 Materials

Laboratory equipment

Access 2 immunoassay	Beckman Coulter
Antigen retrieval citrate bath	Dako
Centrifuge	Beckman Coulter
Cobas e602 autoanalyser	Roche
Cytospin	Shandon
Desktop X-ray microtomograph	Skyscan 1172
Developer	Curix 60, AGFA
Gel plate holder	BioRad
GentleMACS cell dissociator	Miltenyi Biotec
GS-710 Calibrated Imaging Densitometer	BioRad
Incubator	Heraeus Instruments
Inverted fluorescent microscope	Leica DMI 4000B
Inverted light microscope	Leica Leitz DMRB
Mini-Protean Cell	BioRad
Mistral table centrifuge 2000	MSE
MRXII plate reader 4.25	Dynex technologies
Neubauer haemocytometer	Weber
Power pack basic	BioRad
Sample separator (0.75mm/1.5,mm)	The Gel Company
Spectra Max M5 fluorescent plate reader	Molecular Devices
Technico Maxi table centrifuge	Fisher
Thermomixer comfort	Eppendorf
Vortexer	Fisions

Software

CTAN software	CT-analyser Skyscan
Endnote referencing tool	Version 4
Image J software	Version 1.45s
Microsoft Excel	Version 14.2.0
Microsoft PowerPoint	Version 2011
Microsoft Word	Version 14.2.0
Osteomeasure	Osteometrics
PRISM graphpad software	Version 5.0d
SPSS software	Version 19

Plastics and disposables

6 well plate	Corning Inc
96 well plate	Corning Inc
ALZET 2006 sub cut osmotic pumps	Durect corporation
Autoclave tape	3M
Bijoux tubes	Starstedt
Biomax MS film	Kodak

C tubes (gentleMACS)	Miltenyibiotec
Cell scraper	Starstedt
Cell strainer 0.45µm	BD Biosciences
Chamber slides	Thermo Scientific
Conical tubes	BD Falcon
Cytoclips	Shandon
Eppendorf 1.5ml	Starstedt
Filter paper	Sigma-Aldrich
Microscope slides	VWR International
Needles (21, 23, 25 gauge)	Becton Dikinson UK
Non woven sterile swabs	Shermond healthcare supplies LTD
Petri dish	Nunc AS
Pipette tips (10,200,1000µl)	Costar
Polypropylene 6-0 sutures	PROLENE
PVDF transfer membrane	Immmobilon
Reflex 7 surgical clips	Cell point scientific Inc
Strippets (5,10,25ml)	Costar
Surgical Blade	Swann Morton
Syringe filter 0.4µM	Life Sciences
Syringe, 1ml, 5ml, 20ml	Becton Dickinson UL
T75 flask	Corning Inc
Tissue culture flasks, T75	Nalgene Nuc ltd
Universal tube	BD Falcon

Laboratory chemicals and solutions

0.5M Tris-HCL	Geneflow limited/national diagnostics
1.5M Tris-HCL	Geneflow limited/national diagnostics
1% SDS	Biorad
3% H_20_2	VWR chemicals
Acetic acid	VWR chemicals
Acrylamide 30%	Geneflow limited/national diagnostics
Ammonium persulphate	Sigma-Aldrich
Bicinchoninic acid solution	Sigma-Aldrich
Bovine serum albumin	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
Butan-1-ol	Fisher
Caesin	Vector labs
Cell titre 96 Aqueous solution	Promega
Copper sulphate	Sigma-Aldrich
DAB peroxidase substrate	Vector
Dimethyl formaldeyhde	Sigma-Aldrich
Dithiothreitol	Sigma-Aldrich
DMEM media	Gilbco Invitrogen Corp
DMSO	Sigma-Aldrich
Eosin	Merck
Fetal calf serum	Sigma-Aldrich

Fluorescein avidin	Vector
Glycerol	Fisher
Glycine	Fisher
Goat serum	Vector
Haematoxylin	Merck
Horse serum	Vector
HRP detection	Pierce Supersignal West Dura substrate
Mammalian lysis kit reagent	Sigma-Aldich
Methanol	Fisher
Milk powder	Marvel
Napthol	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
Pararosaniline	Sigma-Aldrich
Phosphatase inhibitor	Sigma-Aldrich
Protease inhibitor	Sigma-Aldrich
PVDF membrane	Immobilon
RPMI media 1640 +glutamax	Gilbco Invitrogen Corp
Sodium acetate	Alfa Aesar
Sodium nitrate	Sigma-Aldrich
Sodium Tartrate Dihydrate	Alfa Aesar
Sterile PBS 1x	Gilbco Invitrogen Corp
Supersignal	Pierce
Temed	Sigma-Aldrich
Texas red avidin	Vector
TRIS	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Trypsin EDTA	PAA laboratories
Trypsin enzyme digestion kit	MenaPath
Tween 20	BDH
Vectastain ABC kit	Vector
Vectashield with DAPI	Vector

Cell lines

MCF7	European Collection of Cell cultures
MDA-MB-436	European Collection of Cell cultures
MDA-MB-231	European Collection of Cell cultures
T47D	European Collection of Cell cultures

Antibodies, recombinant proteins and pharmacological products

Amphotericin	PAA
Follistatin rabbit anti human antibody	Novus Biologicals
GAPDH mouse anti human antibody	Abcam
Geranylgeranyl (GGOH)	Sigma-Aldrich
Goat anti-rabbit secondary	Vector
HRP mouse anti-goat secondary	Vector

HRP rabbit anti-goat secondary	Invitrogen
Isofluorane 100% vapor	Abbott
Penicillin/streptomycin	PAA
Pentobarbitone	Animal care Ltd
PhosphoSmad2 (c terminus and linker) rabbit anti human antibodies	Cell signaling
Rapla goat anti human antibody	Santa Cruz
Recombinant human follistatin protein	R&D
Recombinant human inhibin A protein	NIBSC Health protection agency
Recombinant human/mouse activin A protein	R&D
SB 431542	Tocris bioscience
Septrin 40mg/200mg 5ml paediatric suspension	Laboratories genopharm
Smad 2 rabbit anti human antibody	Cell signaling
Vetergesic 0.3mg/ml	Alsto animal health
Zoledronic acid	Novartis

Commercial kits

Human follicle stimulating hormone (FSH) RIA	Roche
Human follistatin ELISA	R&D
Human Inhibin A ELISA	Beckman Coulter
Human Inhibin A ELISA	Cloud-Clone Corp
Human oestradiol RIA	Roche
Human TGFb1 ELISA	R&D
Human, mouse, rat activin A ELISA	R&D
Human/mouse phospho-smad2/3immunoassay	R&D
Mouse follicle FSH ELISA	USCN Life Science
Mouse follistatin ELISA	Cloud-Clone Corp
Mouse inhibin A ELISA	USCN Life Science
Mouse TRAP Assay	Immunodiagnostics systems
Mouse/rat P1NP EIA	Immunodiagnostics systems

2.2 Methods

2.2.1 *In vitro* methodology.

2.2.1.1 Maintenance of cell lines.

Cells were maintained in liquid nitrogen until required. Upon removal from liquid nitrogen they were defrosted by agitating in a water bath maintained at 37°C, and subsequently transferred to a universal container with 9mls RPMI +10% foetal calf serum (FCS). The suspension was centrifuged at 1000rpm for 5 minutes, medium was then removed and the cell pellet re-suspended in required medium prior to transfer into a T75 flask.

Cells were maintained in T75 flasks in incubators at 37 °C, 5% CO_2. Medium was supplemented with 10% FCS. RPMI or DMEM was used dependent upon the experimental design. Cells were split under sterile conditions in positive pressure hoods at confluence. To split cells, firstly they were washed with sterile PBS and following discarding of the PBS, 5mls of trypsin (0.05%) –EDTA (0.02%) added. Flasks were returned to the incubator for a time dependent on the cell line used (typically 5 minutes for MDA-MB-231s and 10 minutes for MCF7s). Detachment from the flasks was confirmed by light microscopy, and 5mls medium added, with the resultant cell suspension pipetted into a universal container to be centrifuged at 1000rpm for 5 minutes. Supernatant was then removed and cell pellet re-suspended in 10mls medium, with a required volume of resultant suspension added to fresh medium in a T75 flask, to create a total volume of 10mls per flask.

2.2.1.2 Cell lines.

MDA-MB-231 and MDA-MB-436 human breast cancer cell lines were used as oestrogen receptor negative (ER–ve) cell lines. Two oestrogen receptor positive (ER +ve) human breast cancer cell lines were used, MCF7s and T47Ds. The choice of cell line was dependent upon the experimental plan, with the majority of experiments carried out on the MDA-MB-231 cells and MCF7 cells.

2.2.1.3 Counting cells using a haemocytometer.

Cell counts were performed using trypan blue and a haemocytometer. Trypan blue is an exclusion dye which is not taken up by cells with an intact cell membrane and thus distinguishes viable cells from dead cells stained blue. A single cell suspension was formed by trypsinising a T75 flask. 10ul of cell suspension was mixed with 10ul trypan blue in an eppendorf. 10ul of the suspension+trypan blue was then pipetted onto the haemocytometer. An inverted light microscope was used to count the number of viable cells in four primary squares. To calculate the number of cells/ml medium the cell count was divided by 4 and multiplied by the volume from which the cell suspension originated. Cell count was then multiplied by 2 to account for the 50% dilution with trypan blue and finally multiplied by 10,000 for final cell count/ml medium. The cell suspension was then centrifuged, and a volume of fresh medium added to create the desired concentration of cell suspension.

2.2.1.4 Cell plating.

Cell count was performed using trypan blue and haemocytometer. The resultant cell pellet was then re-suspended in a volume of media to form a 1x10^6 cells/ml suspension, and the following cell numbers plated dependent on cell type and plate used (cell numbers were determined by time course growth curves performed for each individual cell line in RPMI+10%FCS in 6 or 96 well plates and in chamber slides);

- For experiments using a 6 well plate, 1x10^5 MDA-MB-231 or MDA-MB-436 cells were seeded per well and 2x10^5 MCF7 or T47D cells.
- For experiments using 96 well plates, 1.5x10^3 MDA-MB-231 cells were seeded per well and 3x10^3 MCF7 cells.
- For experiments using chamber slides, 2x10^4 MDA-MB-231 cells per chamber and 4x10^4 MCF7 cells were seeded.

Prior to addition of drug or recombinant protein the cells were allowed to settle in medium+10%FCS for 24 hours. Medium was then removed and replaced by serum free medium to synchronise cell cycle, for 24 hours. The drug/protein of interest was then added in medium containing 10%FCS.

2.2.1.5 Addition of drug and recombinant proteins to cell lines.

After cell plating the following were added to wells dependent on the aim of the experiment. Concentrations of drug, inhibitor and recombinant proteins were chosen based on previously published data. Note all suspensions were sterile filtered before use;

- Zoledronic acid; stock solution (10mM) was diluted to 25μM by adding 12.5μl of stock to 5mls media or 50μM by adding 25ul of stock to 5mls media.

- 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide(SB-431542); Powder was reconstituted with ethanol to 2mM/L stock. 15µl of stock was added to 2850µl 1x PBS to form a 10µm/l solution.
- Recombinant human Inhibin A; Lypophylised powder was dissolved in 1.6mls PBS to form a 3µg/ml stock. A 1µg/ml stock was formed by diluting 100µl of 3µg/ml stock to 200µl PBS. To form a 30µg/ml concentration, 30µl of 3µg/ml stock was added to 2.97mls media. To form a 10µg/ml concentration, 30µl of 1µg/ml was added to 2.97mls media.
- Recombinant human/mouse/rat Activin A; Lypophylised powder was dissolved in 200µl PBS +0.1%BSA to form a 50,000ng/ml stock. 2µl stock was diluted with 18µl media to form a 5000ng/ml stock. 9µl of 5000ng/ml stock was diluted in 4491µl media to make a 10ng/ml stock. Using the 10ng/ml concentration dilutions were made as shown in table 2.1.
- Recombinant human follistatin 315; Lypophylised powder was dissolved in 250µl of PBS+0.1%BSA to form a 100µg/ml stock. 2µl stock was dissolved in 18µl media to form a 10,000ng/ml concentration. 20µl of this concentration was added to 4980µl media to make a 40ng/ml stock. Using the 40ng/ml concentration dilutions were made as shown in table 2.1.2.2.2 *In vitro* sample processing.

2.2.2 *In vitro* sample processing.

2.2.2.1 Cell lysis and protein assay.

Cell suspension was centrifuged at 1000rpm for 5 minutes. Supernatant was discarded and cell pellet re-suspended in 100µl mammalian cell lysis buffer. A further centrifugation step yielded the lysed protein suspension. Phosphatase and protease inhibitors were added as required.

Bicinchoninic protein assay; Bovine serum albumin (BSA) 1mg/ml serves as stock from which serial dilutions form the standard curve. Standard protein concentrations range from 10µg/ml to zero. Standards were prepared by adding 10µl in duplicate to the first 2 columns of a 96 well plate. 10µl of sample was added in duplicate to wells at 100%, 50% and 25% concentration. 200µl BCA reagent was added (1 part $CuSO_4$, 50 parts bicinchoninic acid solution) to each well and the plate incubated for 30 minutes at ambient temperature.

Desired concentration activin/follistatin	Amount of 10ng/ml activin stock / 40ng/ml follistatin stock	Amount of RPMI+10%FCS
6000pg/ml / 24000pg/ml	2700ul	1800ul
1800pg/ml / 7200pg/ml	810ul	3690ul
600pg/ml / 2400pg/ml	270ul	4230ul
180pg/ml / 720pg/ml	81ul	4419ul
60pg/ml / 240pg/ml	27ul	4473ul

Table 2.1 Dilutions of activin (10ng/ml) and follistatin (40ng/ml) for *in vitro* work.

Activin is shown in black and follistatin in blue.

A colour change to purple indicated the presence of protein. This colour change is the result of reduction of Cu2+ions to CU1+ions by peptide bonds in protein. The bicinchoninic acid chelate bind to each Cu1+ ion and form the purple colour. The quantification of amount of purple colour was performed using a microplate reader at wavelength 562nm, with protein levels extrapolated from the standard curve.

2.2.2.2 Protein separation by SDS page and western blot for detecting proteins in cell culture.

The principle of SDS-PAGE is that proteins which have been denatured by sodium docecyl sulfate (SDS) can be separated by an electric field using a polyacrylamide gel electrophoresis (PAGE). SDS is negatively charged and binds to polypeptide chains in differing amounts relative to the molecular mass. The negatively charged proteins are attracted to a positively charged anode. The gel separates the proteins according to molecular mass with smaller proteins travelling further down the gel compared to larger proteins. Following protein separation the samples can be further processed to Western blot to probe for specific proteins of interest using antibodies.

Proteins were denatured by adding 1ml of sample buffer (10ml 0.5M Tris, SDS 2g, 6.4ml glycerol, bromophenol blue) to 25mg dithiothreitol and adding 100μl of resultant mixture to 300μl protein sample. The sample was heated at 95^{o}C for 5 minutes and then transferred to ice.

12% separating gels and stacking gels were formed by addition of the following substrates in the volumes shown in table 2.2.

10μl of denatured protein was added to each well plus a molecular weight marker. Gels were run in running buffer (0.25M Tris, 0.2M glycine, 1% SDS, distilled water 500ml) at 200V for 1 hour to ensure adequate separation of proteins according to mass. Gels were then equilibrated with methanol activated PVDF membrane, filter paper and pads and transferred in transfer buffer (0.25M Tris, 0.2M glycine, distilled water 500ml) at 70V for 70 minutes. Membranes were then removed and blocked in appropriate blocking solution for 1 hour at ambient temperature. Primary antibodies were then added and incubated overnight at 4^{o}C.

Table 2.2. Substrates and volumes required to form a 12% separating gel.

	Separating Gel	Stacking gel
Distilled water	3.35ml	6.1ml
1.5M Tris-HCL pH 8.8	2.5ml	-
0.5M Tris-HCL pH 6.8	-	2.5ml
10%SDS	100µl	100µl
30% Acrylamide	4ml	1.3ml
20%APS	50µl	100µl
TEMED	5µl	10µl

After discarding the primary antibody, membranes were washed x3 in PBS-tween. Secondary antibodies were then added in blocking solution for 1 hour (see table 2.3). Completion of the experiment involved x5 washes and addition of chemiluminescence with exposure of membranes, in the dark room, to photographic film with subsequent automated development of the film.

2.2.2.3 Enzyme linked immunoabsorbance assays (ELISAs) for quantification of protein levels in cell culture supernatant.

The principle of an ELISA is as follows:

Antibodies to the protein of interest coat each well of a 96 well plate. Sample is then added and the specific protein within the sample binds to the immobilised antibody. Addition of a further detection antibody binds the antibody:protein complex. The detection antibody is then bioconjugated to a second antibody linked to an enzyme. Addition of an enzyme substrate at the end of the assay results in a colour change to produce a visible signal that can be quantified using a plate reader at specific absorbance.

> Human Follistatin (FS288, FS300, FS315); Follistatin is a paracrine glycoprotein expressed in many tissues. It binds the protein activin and neutralises its biological activity, it also inhibits other members of the TGFβ superfamily of signaling proteins. The follistatin ELISA was carried out according to manufacturers instructions. Standards were mixed by diluting 160,000pg/ml follistatin stock with calibrator diluent RD5-21 to form serial dilutions ranging from 16,000pg/ml to zero. 100μl of assay diluent was added to each well which was pre-coated with antibody specific for follistatin. 100μl of standard or sample was added per well in duplicate. The plate was incubated at 4^{o}C for 3 hours. Following incubation the plate was washed x4 using a squirt bottle. 200μl of follistatin conjugate was added to each well and the plate incubated at 4^{o}C for a further 2 hours. Washing was repeated and 200μl of substrate solution was added with the plate maintained in darkness for 20minutes. Final steps involved addition of 50μl stop solution to each well and the optical density was read at 450nm using a microplate reader. Follistatin levels were determined from the standard curve. Mean minimum detection limit is 29pg/ml (10-83pg/ml). All intra-assay CV's were below 15%.

Table 2.3 Antibodies and concentrations used for western blotting

Antigen	Gel (%)	Primary antibody	Secondary antibody
Gap DH	12	Mouse polyclonal antibody 1:20,000	Goat anti-mouse HRP conjugated 1:15,000
Rap1a	12	Goat polyclonal antibody 1:200	Rabbit anti-goat HRP conjugated 1:30,000
Phospho-Smad2 (Ser 245/250/255)	12	Rabbit polyclonal antibody 1:1000	Goat anti-rabbit HRP conjugated 1:8000

Gap DH and Rap1a concentration was chosen based on previous experience within the laboratory group. Phospho-Smad2 concentration was optimised from suggested doses in the product literature.

- Human/Mouse/Rat Activin A; Activin A is a paracrine protein involved in cell proliferation, apoptosis and differentiation and is a member of the TGFβ superfamily of signaling proteins. The activin A ELISA was carried out as follows. The activin A kit was carried out according to manufacturers instructions. 200μl of activin A biotinylated antibody was added to all wells and the plate incubated on an orbital shaker set at 500rpms for 15mintes. Standards were formed from dilutions of the activin A standard with levels ranging from 1000pg/ml to zero. 100μl of assay diluent RD1-98 was added to each well. 100μl standard or sample was added to each well in duplicate. The plate was incubated at ambient temperature on the orbital shaker for 3 hours. Following this the plate was washed x6 using wash buffer and a squirt bottle. 200μl of activin A conjugate was added to each well. The plate was incubated at ambient temperature for 1 hour on the shaker. Wash steps were repeated x6. 200μl of substrate solution was added to each well and incubated for 30 minutes at ambient temperature in darkness. Final steps involved addition of stop solution to each well and the plate was read on a microplate reader at 450nm. Activin concentrations were extrapolated from the standard curve. Mean minimum detection limit was 3.67pg/ml (0.75-7.85pg/ml) and intra-assay CVs were <15%.

- Human TGFβ1*;* TGFβ1 is a paracrine protein of the TGFβ superfamily of proteins involved in cell differentiation, proliferation and apoptosis. The TGFβ1 ELISA was carried out as follows. Latent TGFβ1 was activated by adding 20μl of 1N HCL to 100μl of cell culture supernatant. Following mixing and incubation for 10 minutes at ambient temperature 20μl of 1.2N NaOH/0.5M HEPES was added and the sample processed for ELISA. Standards were formed by diluting the 2000pg/ml standard with dilutions ranging from 2000pg/ml to zero. 50μl of assay diluent RD1-1 was added to each well that was coated with a monoclonal antibody specific for TGFβ1. 50μl of standard or activated sample was added to each well in duplicate. The plate was incubated at ambient temperature for 2 hours. The plate was then washed x4 with wash buffer using a squirt bottle. 100μl of TGFβ1 conjugate was added to each well and followed by a further incubation for 2 hours at ambient temperature. Washing was repeated x4 and

100μl of substrate solution was added to each well and incubated in darkness for 30 minutes at ambient temperature. Final steps involved addition of 100μl of stop solution to each well and the plate was read on a microplate reader at 450nm. TGFβ1 levels were extrapolated from the standard curve. Mean minimum detection limits were 4.61pg/ml (1.7-15.4pg/ml) and intra-assay CVs were <15%.

2.2.2.4 Cell titre Aqueous One solution cell proliferation (MTS) assay for assessing viable cell number in cell culture.

The MTS proliferation assay is a colorimetric assay for determining the number of viable cells. The active compound is [3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt:MTS]noon and an electron coupling reagent (phenazine ethosulfate;PES). The MTS compound is bioreduced by cells into a coloured formazan product via NADH produced by mitochondrial dehydrogenase enzyme in metabolically active cells. The coloured compound is soluble in medium and is directly proportional to the number of viable cells and can be quantified by recording absorbance at 490nm on a plate reader. 1.5×10^3 MDA-MB-231 or 3×10^3 MCF7 cells were seeded per well of a 96 well plate in RPMI +10%FCS, leaving 2 columns blank for medium+10%FCS only controls. Background absorbance from medium+10%FCS with or without protein was within quoted range for assay (0.2-0.3 absorbance units). Plates were incubated overnight and the following day medium discarded and replaced by serum free RPMI to ensure synchronization of cell cycle for 24 hours. Recombinant proteins; human activin and human follistatin or the ALK 4/5 inhibitor SB-431542 were then added to the wells at differing concentrations in RPMI+10%FCS and medium+protein was replaced every 24 hours. The plates were incubated for the required time-points, and at the end of the experiment 20μl of CellTitre96Aqueous One solution was added to control, treatment and media only wells. The plate was incubated in the darkness for 3 hours and then analysed using a plate reader with absorbance at 490nm (Fig 2.1)

2.2.2.5 Immunofluorescence to quantify levels, and visualise localisation, of proteins in cell culture.

Cell based fluorescent ELISA; This assay uses fluorogenic substrates to quantify levels of human/mouse/rat phospho-Smad2/3 in whole cells. Smad2/3 are membrane bound intracellular proteins that are phosphorylated in response to

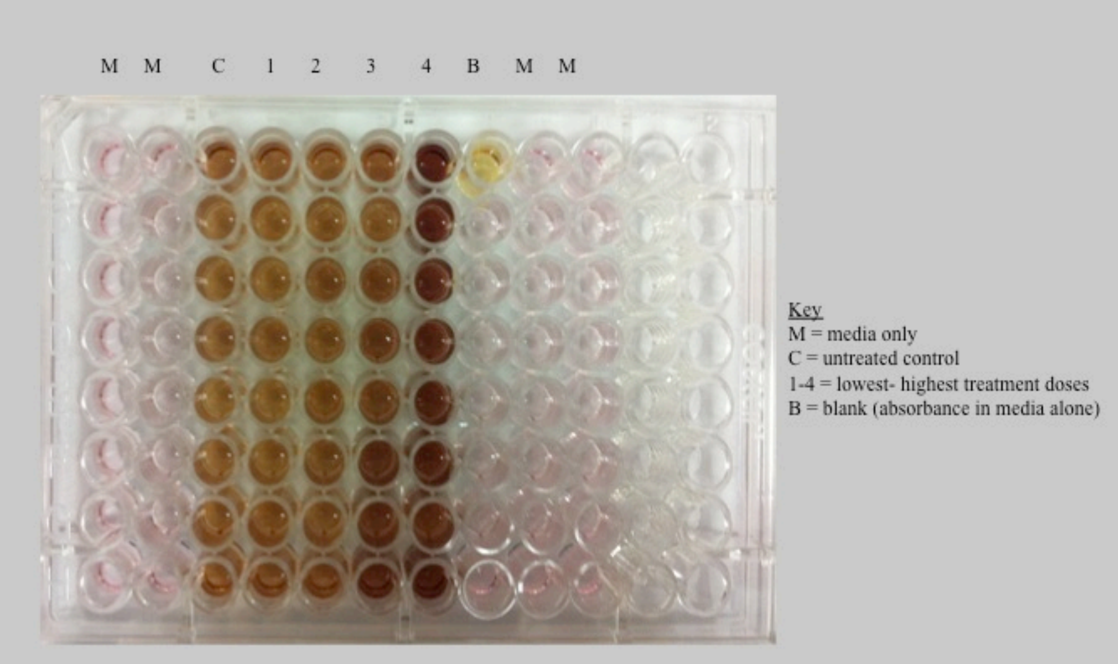


Figure 2.1. Representative photograph of an MTS assay.

96 well plates were seeded with tumour cells and treated with increasing doses of recombinant protein. MTS solution was added at the end of the experiment and broken down to a coloured product that can be quantified by use of a plate reader.

activin binding to its ACRII cell surface receptor. The principles of the assay are detailed in Figure 2.2 and are summarised as follows: MDA-MB-231 cells were seeded at a density of $1.5x10^3$ cells per well of a 96 well plate in RPMI+10%FCS. Cell were left for 48 hours at 37°C in a cell incubator. After 48 hours media was replaced by serum free medium for a further 24 hours incubation. Following this cells were treated for 1 hour with drug/protein/conditioned medium of interest followed by fixation with 100μl of 4% formaldehyde in 1XPBS for 20 minutes. 3 wash cycles followed this and then cells were incubated in quenching buffer for 20 minutes at ambient temperature. After further wash cycles cells were blocked with blocking buffer for 1 hour. Cells were then washed x3 and incubated with 100μl of primary antibody overnight at 4°C. Following further washing, primary antibody was removed and 100μl of secondary antibody was added for 2 hours. The last step involved addition of 2 fluorescent substrates for 60 and 40 minutes each, and the plate was read using a fluorescent plate reader with excitation at 540nm and emission at 600nm to identify levels of phosphorylated Smad2/3, and excitation at 360nm and emission at 450nm to represent the levels of total Smad2/3.

- To visualise localisation of phospho-Smad2/3 in whole cells, chamber slides were used to grow MCF7 and MDA-MB-231s in cell culture. $4x10^4$ MCF7 cells and $2x10^4$ MDA-MB-231 cells were seeded per chamber well in RPMI+10%FCS for 24 hours, then medium was replaced by serum free RPMI for 24 hours. Each well was then treated with recombinant protein or drug in RPMI+10%FCS, and chambers maintained in incubators for required duration. Medium was then discarded and 4% paraformaldehyde (PFA) added to chambers for 4 hours at 4°C. Following fixation, chambers were washed x2 in PBS and blocking solution added (5% goat serum in PBS) for 1 hour at ambient temperature. Chambers were then incubated overnight at 4°C with primary antibody (phosphorylated Smad2 1:100) in 5% BSA, ensuring one chamber was maintained as a negative control. After 24 hours, x4 washes preceded addition of secondary antibody (goat anti-rabbit 1:100) in antibody solution (5% goat serum in PBS) for 1 hour at ambient temperature. Washes were then repeated and fluorescine avidin in 10mM HEPES+0.15NaCl was added for 30 minutes at ambient temperature. Chambers were then dismantled, and the slide mounted with mounting medium containing

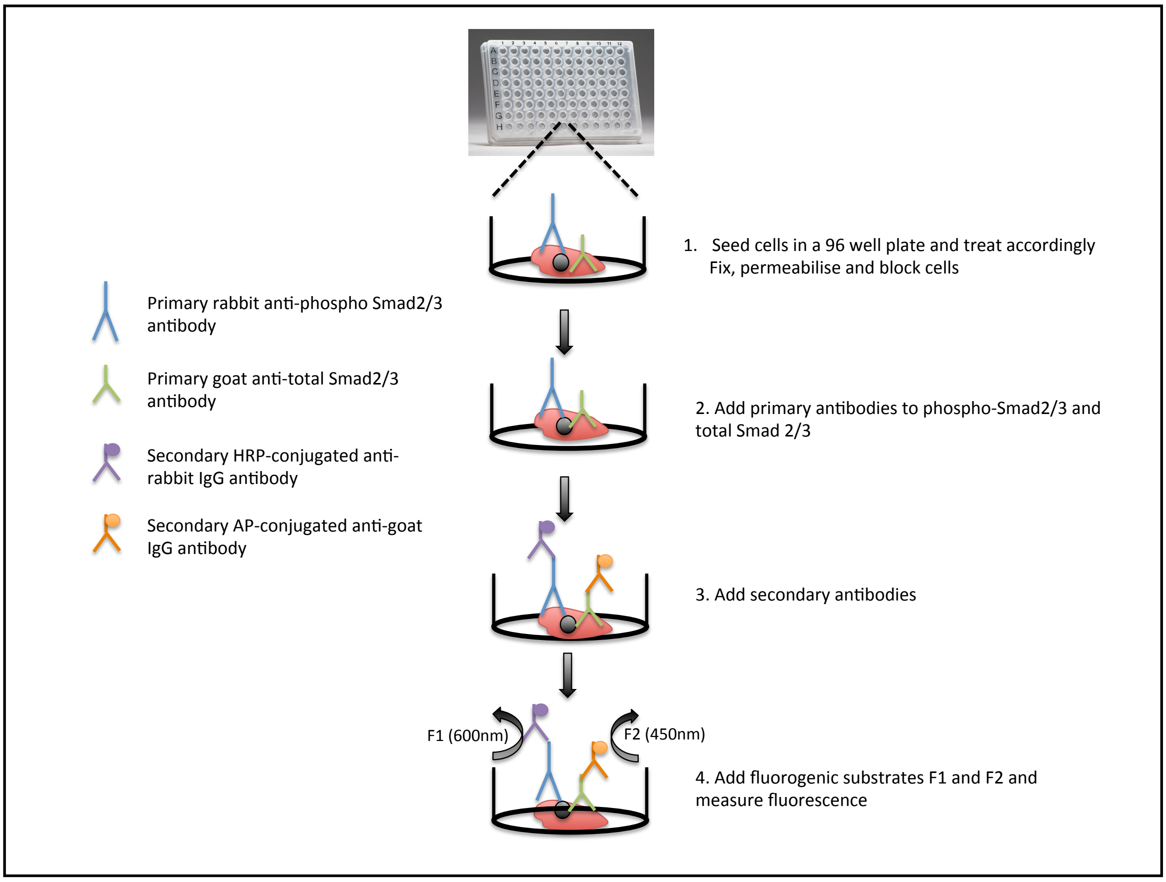


Figure 2.2 Principles of a cell based ELISA for quantification of phospho-Smad2/3 and total Smad2/3 levels in whole cells.

MDA-MB-231 cells were seeded in 96 well plates in RPMI+10%FCS. After 48 hours medium was replaced by serum free RPMI. After 24 hours cells were treated for 1 hour with protein/drug/supernatant followed by fixation and detection of Smad2/3 as per protocol.

DAPI and cover slips attached. Slides were imaged using an inverted fluorescent Leica microscope to evaluate localisation of pSmad2 protein. Localisation was determined by imaging a minimum of 100 cells per chamber, and counting number of cells with fluorescent protein in the nucleus (identified by DAPI), and expressing the result as a percentage of total number of cells counted (see chapter 4 for further details).

2.2.3 *In Vivo* methodology.

2.2.3.1 Ethics and home office licensing.

All experiments were carried out at the University of Sheffield Biological Service, under the Home Office Project License number 40/3531. Individual study plans were reviewed by the project license holder, prior to commencement, to ensure adherence to the guidelines set out in the license. Female balb/c nude mice were obtained from Leeds University breeding program and Charles River. Animals were 12-weeks old at commencement of experiments.

2.2.3.2 Anaesthetic.

Where recovery of the animal was planned, isofluorane was the gaseous anesthetic of choice. Individual mice were placed in an induction chamber with oxygen flow set to 4L/min and isofluorane flow set to 5%. Once mice were sedated they were removed from the induction chamber, isofluorane flow turned down to 3% and delivered by a nose cone. 50μl of vetergesic was administered sub-cutaneously, and once pedal reflex was lost the procedure was commenced. Animals were placed in an incubator post procedure, and observed until alert and mobile before returning to cages.

Where terminal sedation of the animal was planned, 100μl pentobarbitone was injected intraperitoneal. Once pedal reflex was lost the terminal procedure was commenced.

2.2.3.3 Insertion and loading of sub-cutaneous ALZET osmotic pumps.

ALZET 2006 osmotic pumps were used to deliver recombinant human inhibin A protein in 1x PBS or 1xPBS alone. The pumps were provided sterile, and all handling and loading of the pumps was performed under sterile conditions in a cell hood according to manufacturers instructions. Figure 2.3 demonstrates the structure of the ALZET pumps.

To calculate the concentration of recombinant inhibin A to add the following formula was used:

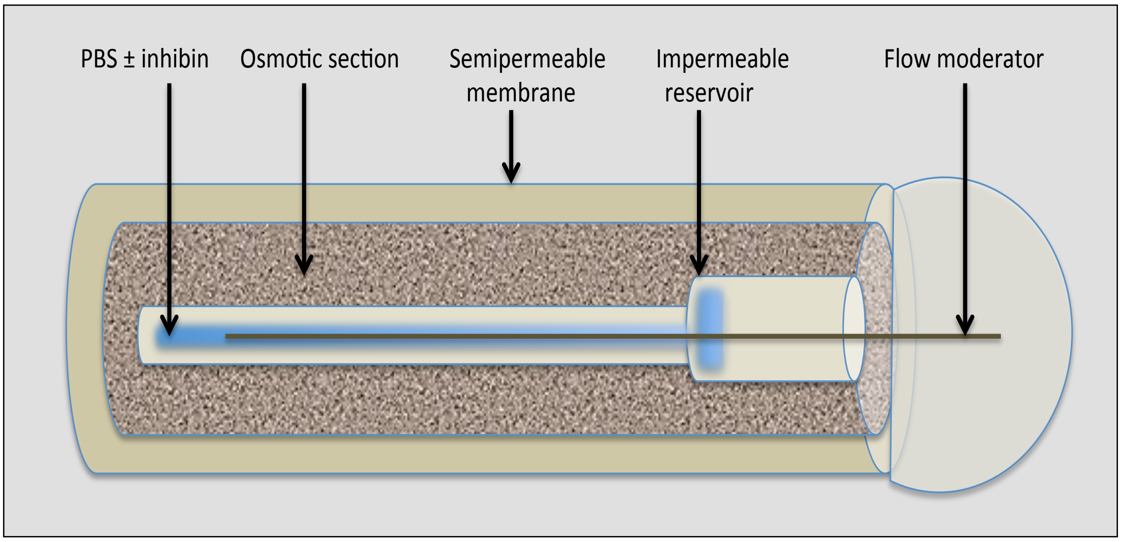


Figure 2.3. Schematic of an ALZET pump.

The 2006 model delivered a continuous solution of PBS+/- recombinant human inhibin protein for a maximum of 42 days in the mice, negating the need for multiple injections.

$$Cd = \frac{K0}{Q}$$

Cd = concentration of drug required (μg/μl), K0 = mass delivery rate (μg/hr), Q = volume delivery rate (set at 0.15μl/hr in the 2006 model).

To deliver 120ng/day inhibin A (5ng/hr), 33.4ng/μl of inhibin A was required per pump. To deliver 60ng/day inhibin A (2.5ng/hr), 16.7ng/μl of inhibin A was required per pump, To deliver 10ng/day inhibin A, 2.8ng/μl was required per pump. Pumps were primed so that they were immediately active on the day of surgery by placing the loaded pumps in sterile 0.9% saline at 37°C for 60 hours prior to surgery.

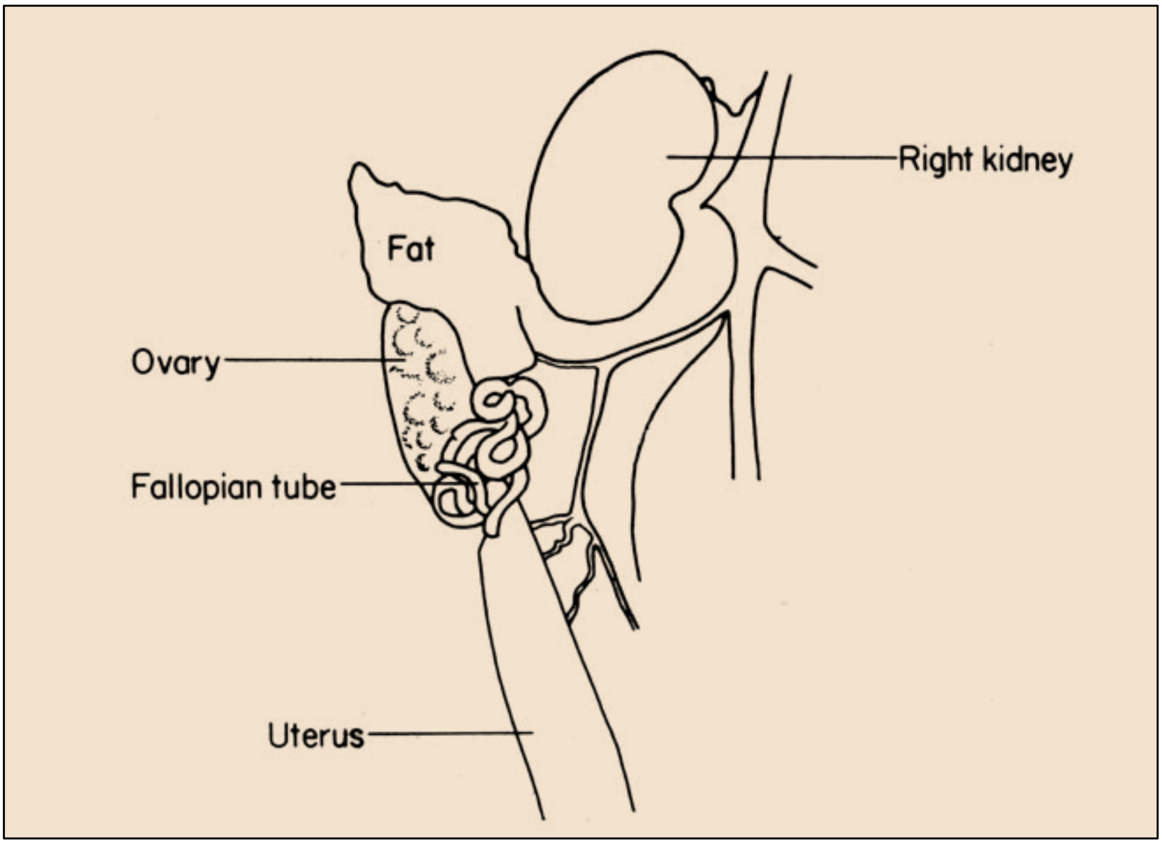
To insert the pumps, mice were anaesthetised as per recovery method, the operating field was maintained under sterile conditions using chlorhexidine, and the mouse positioned in a prone position. A small skin incision was made using a scalpel in the ventral mid line, overlying the lumbar vertebrae. The skin was freed from underling connective tissue by blunt dissection, and the ALZET pumps inserted sub-cutaneously. The wound was closed with surgical clips or non-absorbable 6-0 surgical sutures, which remained in place for 7 days prior to removal (Figure 2.4). Mice were weighed post pump insertion to record a baseline weight, and placed in an incubator until recovery.

2.2.3.4 Ovariectomy of mice.

Mice were anaesthetised as per recovery method and placed in prone position in a sterile operating field. A sub-cutaneous incision was made in the same area as for the pump insertion. Entry to the right side of the posterior peritoneal cavity was obtained by blunt dissection. Following this the right ovary was identified from surrounding peri-renal fat and fallopian tube, and externalised (Figure 2.5). The ovary was then removed from the fallopian tube by gentle shearing, which prevents haemorrhage. The same procedure was carried out on the left side via the same incision point. A sham procedure involved externalisation of both right and left ovaries, followed by return to the peritoneal cavity. The sub-cutaneous incision was closed using prolene 6.0 non absorbable sutures. Septrin paediatric suspension was added to a litre of water and divided among the cages to prevent infection. Mrs Anne Fowles assisted with training for the ovariectomy (OVX) and sham procedures.

Figure 2.4. Representative image of an ALZET pump *in vivo*.

ALZET pumps were placed sub-cutaneous under isofluorane anaesthetic with insertion point sutures for wound closure. Black arrows highlighting side and front view of the pumps.



Reproduced from The Anatomy of the laboratory mouse. Margaret J Cook. Elsevier Academic Press 1965. Adapted for web by Mouse Genome Informatics, The Jackson Laboratory, Bar Harbour, Miane, 2008.

Figure 2.5. Murine anatomical position of the ovary.

Mice were anaethetised and placed in the prone position. An incision was made at the level of the iliac crests in the mid line, and following blunt dissection into peritoneal cavity, peri-renal fat was used to localise ovaries. Ovaries were removed by gentle shearing and peritoneal cavity closed.

2.2.3.5 Exsanguination from the heart at termination of experiment, and terminal sample collection.

Following administration of pentobarbitone for terminal sedation, pedal reflex was checked to ensure deep sedation. With the mouse in the supine position, using a 1ml syringe and 25-gauge needle, the xiphisternum was identified and the needle introduced towards the direction of the left shoulder. Cardiac blood was collected slowly to prevent collapse of the heart, with an average volume of 800μl. Blood was left at ambient temperature to clot, then spun at 4000rpm for 15 minutes. Serum layer was removed and placed in a 1.5ml tube for storage at -20°C until processing. Following cervical spine dislocation, tissues of interest were dissected free and processed as follows:

- Hind limbs were placed in 4% PFA at 4°C, and changed to 10% EDTA after 48 hours. This solution was changed every 2 days for 2 weeks to ensure adequate decalcification of the bone prior to processing.

- Calvaria were dissected free of soft tissue and the edges cut to remove debris. They were individually crushed in 3mls of 1x PBS. The cell suspension was then filtered using a cell strainer, to remove bone debris, and a cell count performed using trypan blue. The cell suspension was centifuged at 1500rpm for 5 minutes and 300μl of resultant supernatant taken for processing to ELISA or frozen at -20°C.

- Liver was divided and either placed in 4% PFA for 48 hours at 4°C, then changed to 70% ethanol until processing for histology, or was weighed and homogenised to allow quantification of protein via ELISA. Homogenisation of liver was performed using a cell dissociation program (M_spleen_01_04) on the GentleMacs tissue homogeniser. Briefly, liver was placed in a C tube with 9mls PBS. The C tube was attached to the homogeniser and run for 45 seconds. Following this, the suspension was centrifuged at 7000rpm for 5 minutes and supernatant transferred to an eppendorf. 2 further centrifuge steps were carried out at 7000rpm for 5 minutes to ensure clear supernatant and all debris removed. Supernatant was frozen at -80°C for processing to ELISA.

2.2.4. *In vivo* sample processing

2.2.4.1 Blood analysis

Enzyme linked immune absorbance assay (ELSIA) for quantification of protein levels in serum:

- Mouse Follicle stimulating hormone (FSH); FSH is a pituitary secreted hormone, which is under negative feedback from the ovarian secreted hormone inhibin A. The ELISA kit was carried out according to manufacturers instructions. Serum was defrosted and agitated, no dilution of samples was required. Standards were mixed by diluting 180ng/ml FSH stock with standard diluent to form serial dilutions ranging from 180ng/ml to zero. 100µl of standards or sample were added to each well in duplicate and incubated for 2 hours at 37^{o}C. Liquid was removed from wells and 100µl of detection reagent A was added followed by a 1 hour incubation at 37^{o}C. Liquid was removed from wells and x4 wash steps performed using a squirt bottle. 100µl of detection reagent B was added to wells and incubated for 30 minutes at 37^{o}C followed by x5 wash steps. Following this 90µl of substrate solution was added for 20 minutes at 37^{o}C in the dark, followed by the final step of addition of 50µl of stop solution to the wells. The optical density was read at 450nm using a microplate reader, and FSH levels were determined from the standard curve. Mean minimum detection limit was 0.95ng/ml. All intra-assay CV's were below 15%.

- Mouse Tartrate Resistant Acid Phosphatase (TRAP); TRAP is produced by bone resorbing osteoclasts and can be detected in serum. EIA was carried out as follows and according to manufacturers instructions. Serum was defrosted and agitated. 100µl of anti-mouse TRAP antibody was added to each well of the microplate and incubated for 60 minutes at ambient temperature on a microplate shaker set to 950rpm. Wells were washed x3. 100µl of calibrator or control was added to appropriate wells in duplicate and 75µl of 0.9% NaCL followed by 25µl of sample was added to wells in duplicate. 25µl of releasing agent was added to all wells and the plate incubated at ambient temperature for 60 minutes shaking at 950rpm. The plate was washed x3 and 100µl of substrate solution added to all wells. An incubation step followed for 2 hours at 37^{o}C followed by addition of 25µl stop solution to all wells. The plate was read at 405nm using a microplate reader. Mean minimum detection limit was 0.1U/L. All intra-assay CV's were below 15%.

- Mouse/rat P1NP; P1NP is a marker of bone formation and can be detected in serum. The P1NP EIA was carried out according to manufacturers instructions. Serum was defrosted and agitated, 50μl of calibrator or control was added to appropriate wells in duplicate to an antibody coated plate. 5μl of sample and 45μl sample diluent was added to each well. Following this 50μl of P1NP biotin was added to each well and the plate was incubated at ambient temperature on a microplate shaker at 500rpm for 1 hour. Following this x3 wash steps were performed, and 150μl of enzyme conjugate added to wells, followed by a 30 minute incubation at ambient temperature. After a further x3 wash steps, 150μl of TMB substrate was added to all wells and incubated at ambient temperature for 30 minutes. 50μl of stop solution was then added and the absorbance read at 450nm using a microplate reader. The optical density was read at 450nm using a microplate reader, and P1NP levels were determined from the standard curve. Mean minimum detection limit was 0.7ng/ml. All intra-assay CV's were below 15%.

- Mouse Inhibin A; Inhibin A is secreted by ovaries throughout reproductive life span. The ELISA kit was already optimised and was therefore carried out according to the manufacturers instructions. Serum was defrosted and agitated, 100μl of standard or sample was added to each well in duplicate and incubated for 2 hours at 37^{o}C. 100μl of detection reagent A was added to each well and incubated at 37^{o}C for 1 hour. x3 wash cycles were followed by addition of 100μl of detection reagent B for 30 minutes at 37^{o}C. A further 5 wash cycles preceded addition of 90μl of substrate solution for 20 minutes with the final step being addition of 50μl of stop solution and the plate was read at 450nm. Mean minimum detection limit was 6.1pg/ml. Intra- assay CV's were below 15%.

- Human Inhibin A ELISA; to detect recombinant human inhibin A protein in mouse serum a human specific ELISA was used. The ELISA was carried out according to manufacturers instructions. 100μl of sample or standard was added to each well in duplicate. Liquid was removed and 100μl of detection reagent A was added immediately and incubated at 37^{o}C for 1 hour. Wells were washed 3 times and 100μl of detection reagent B followed by an incubation period of 1 hour at 37^{o}C. 5 further washes were then performed, and 90μl of substrate solution added for 20 minutes at 37^{o}C. 50μl of stop solution was added and the plate was read at 450nm. Mean minimum detection limit was 39pg/ml. Intra-assay CV's were below 15%.

2.2.4.2 Analysis of tibia.

Microcomputed tomography (μCT).

Microtomography uses X-rays to create a 3D image that can be used to analyse tissue architecture. The proximal right tibia was used for evaluation of bone architecture. Activation of the X-ray source was performed using the Skyscan 1172 program. The tibia was placed proximal end down in a secure container sealed with cling film, and camera set to medium resolution of 2000x1024. The height of the sample was adjusted to include the region of interest (ROI). The x-ray source was sealed and each bone scanned individually. Images were then reconstructed using NRecon software to form a series of JPG cross sectional images for each bone, 4.4μm apart over a distance of 1.0mm. To analyse the images, a reference point was defined to ensure the same section of each bone was analysed. The reference point is shown in figure 2.6 and bone was analysed from this point over a distance of 1.0mm. The ROI of each cross sectional image was selected by drawing around the inside of the cortical bone, to encompass the trabeculae (Figure 2.7). This process was repeated several times over the length of the tibia to maintain the ROI within the cortex of bone. Using analysis software (CT_{AN}) the ROIs for each bone were reconfigured to give a value of bone volume to tissue volume percentage (BV:TV) which could be compared between groups.

Immunohistochemistry:

TRAP stain; TRAP is an enzyme expressed by osteoclasts and can be used to identify bone cells on tissue sections. To perform the stain acetate buffer was made by dissolving 5.44g sodium acetate in 200ml distilled water and adding 50ml 1.2% acetic acid (494ml H_2O+6ml

acetic acid) and 4.6g sodium tartrate. This solution was incubated at 37°C for 2-4 hours. Sections were dewaxed by placing in xylene and rehydrated with alcohols. This was

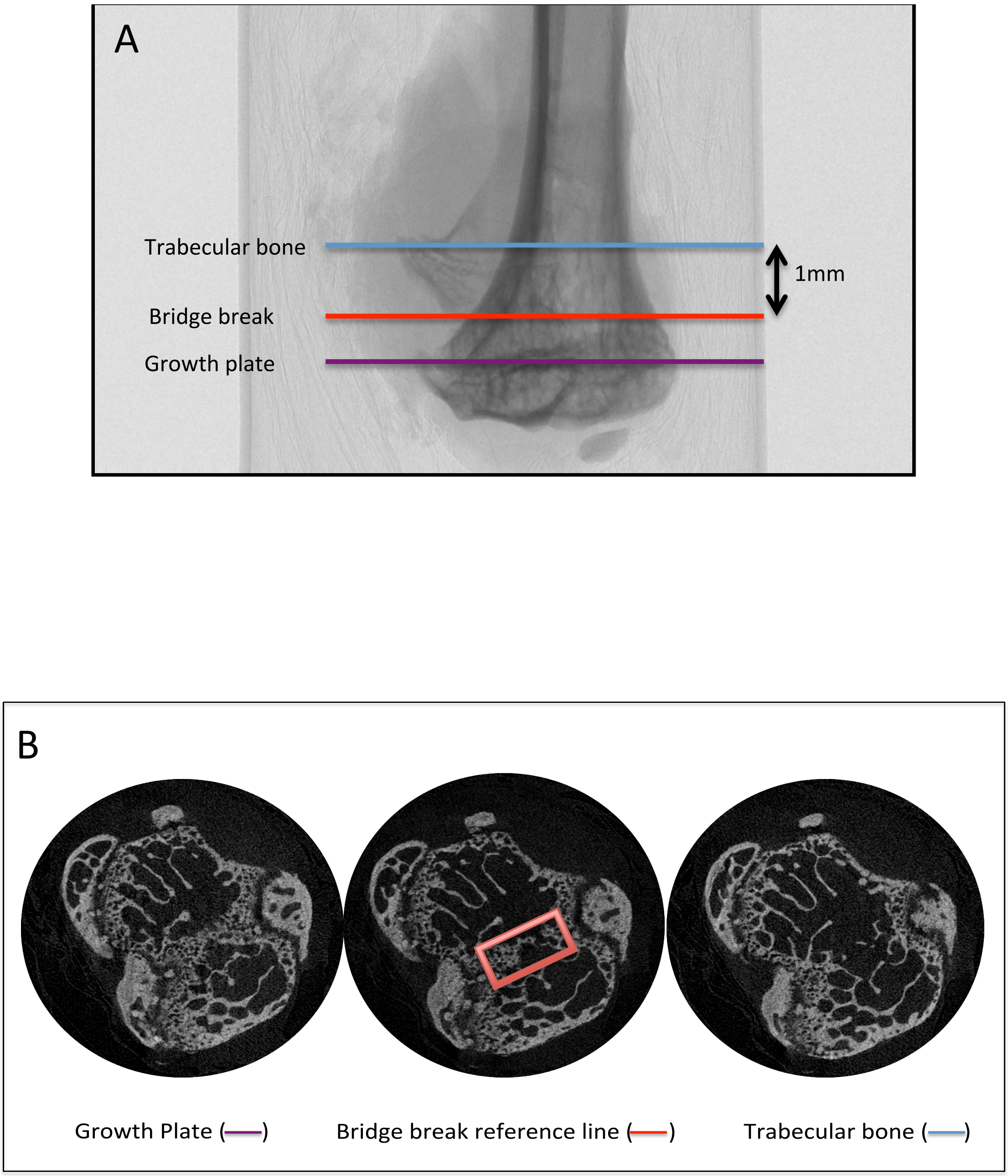


Figure 2.6 Representative image of cross sectional images of bone at increasing distances from the growth plate.

A) µCT image of tibia with corresponding reference points highlighted on cross sectional imaging in B).

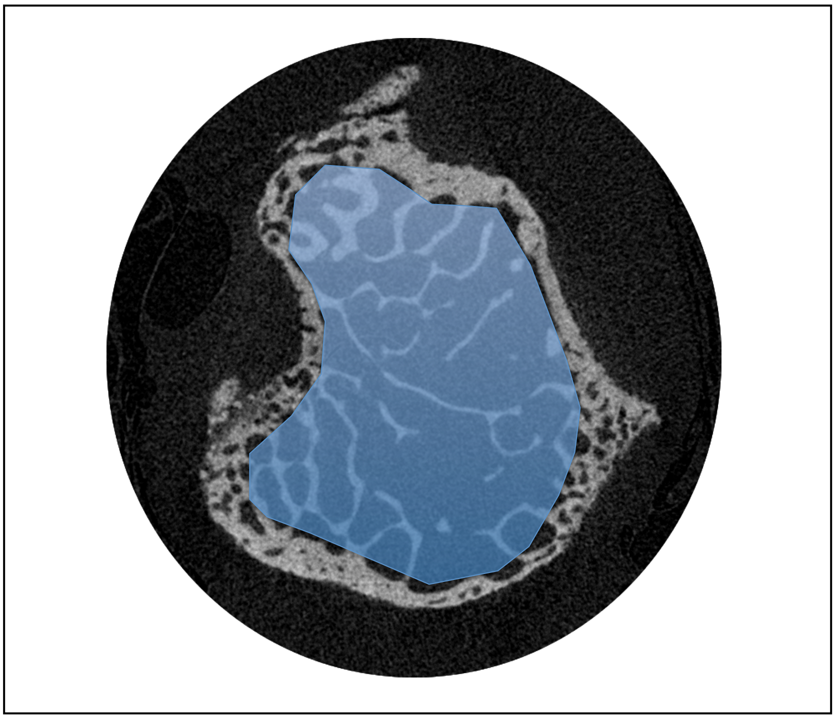


Figure 2.7. Region of interest (ROI) for analysis of trabecular bone volume

ROI was defined by identifying the bridge break in the growth pate and analysing trabecular bone volume over a distance of 1.0mm above bridge break. ROI is represented by blue shading.

followed by placing sections in warmed acetate buffer for 5min at 37°C. Sections were then incubated in solution A; 0.02g napthol phosphate plus 1ml dimethyl formaldehyde and 50ml of acetate buffer, for 30 minutes at 37°C. Sections were then incubated insolution B; 0.08g sodium nitrite in 2ml distilled H_2O, plus 2ml pararosaniline. 2.5ml of this solution was added to 50ml acetate buffer and sections were incubated for 15 minutes at 37°C. Sections were rinsed and counterstained with haematoxylin for 20 seconds, then subsequently dehydrated and mounted with cover slips.

Osteomeasure scoring of slides:

Osteomeasure software allows quantification of bone cells from TRAP stained tissue sections. Osteoclasts were identified by the pink stain visable after TRAP staining. Osteoblasts were identified according to morphology and location. To be identified as an osteoblast the cell was characterised as being cuboidal in shape with a nucleus orientated to one end of the cell. It also had to be in contact with the trabecular surface of the bone, and with another cell of similar appearance. See figure 2.8. The microscope was set at 10x objective and analysis options selected to include total surface measurement, osteoclast number and surface and osteoblast number and surface. The growth plate was identified and analysis started 125μm below this reference point. The entire trabecular surface was analysed, and summary data recorded at the end of each analysis. Results were expressed as number of each bone cell per mm trabecular surface.

2.2.4.3 Analysis of Calvaria

Enzyme linked immune absorbance assay (ELISA) for quantification of protein levels in calvaria;

- Mouse activin ELISA were carried out as previously described in sections 2.2.2.3

- Mouse follistatin ELISA; The follistatin ELISA was performed according to the manufacturers instructions. Briefly 50μl of standard or sample was added to each well in duplicate followed by immediate addition of 50μl of detection reagent A. The

  plate was incubated for 1 hour at 37°C. x3 wash cycles were then performed followed by addition of 100μl of detection reagent B. A further incubation for 30 minutes at 37°C followed and then a further 5 wash cycles. 90μl of substratesolution was then added for 20 minutes at 37°C, and 50μl of stop solution

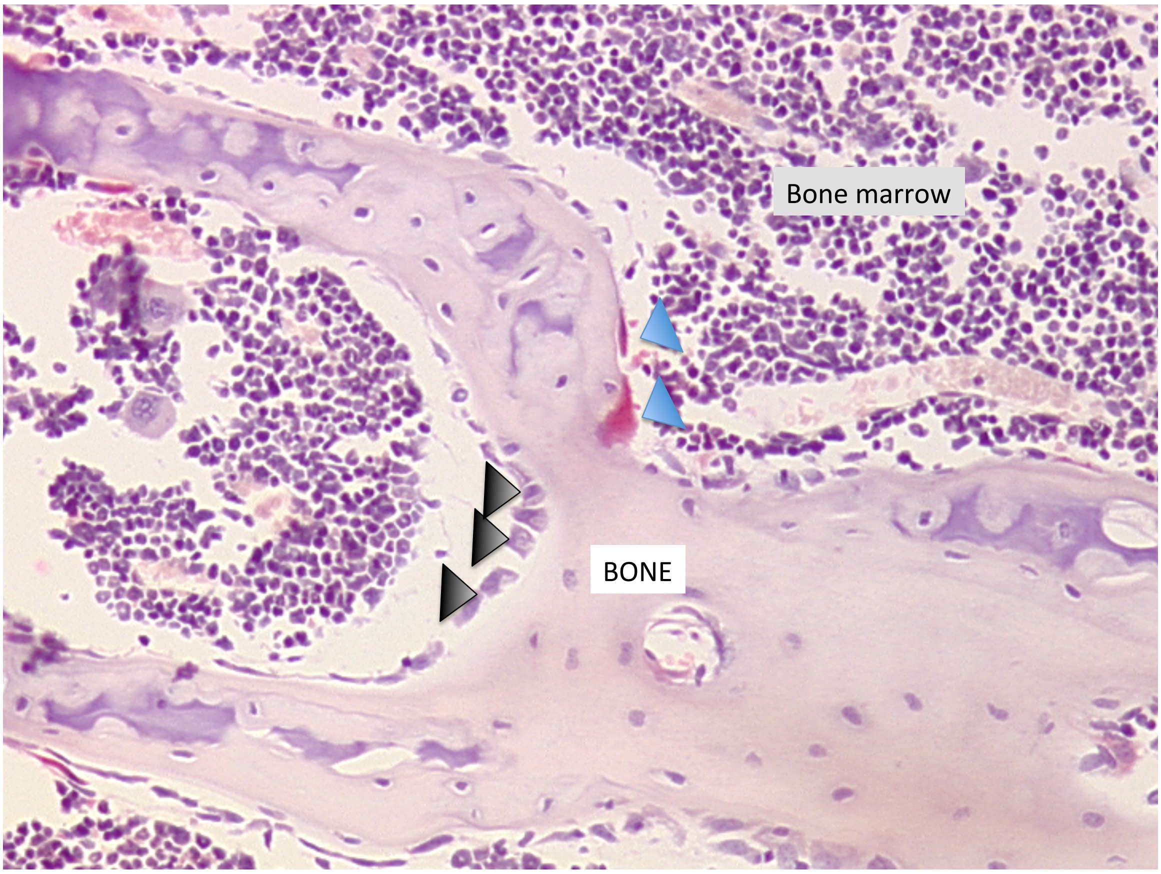


Figure 2.8. Identification of osteoblasts and osteoclasts using TRAP stain.

Osteoblasts were identified by their cuboidal shape and location on the surface of the bone and in association with another cell of the same type (black arrows). Osteoclasts were identified by their pink TRAP +ve staining and on the surface of the bone (blue arrows) (x20 magnification) was added prior to reading the plate at 450nm. Mean minimum detection limits were 0.94ng/ml

2.2.4.4 Analysis of Liver

Enzyme linked immune absorbance assay (ELISA) for quantification of protein levels in liver;

- Mouse activin ELISA were carried out as previously described in sections 2.2.2.3
- Mouse follistatin ELISA were carried out as previously described in section 2.2.4.3

Immunohistochemistry:

Haematoxylin and eosin stain (H&E); H&E is a common stain used to evaluate morphology of various tissues. The haematoxylin stains nuclei blue and the eosin stains eosinophilc structures such as cytoplasm pink. To perform the stain samples were dewaxed in xylene and rehydrated with alcohols, followed by a rinse step in water for 10 seconds. Sections were then submerged in Gill's haematoxylin for 2 minutes and rinsed until clear. They were then submerged in 1% eosin for 5 minutes before being rinsed, dehydrated, mounted and visualized with a light microscope.

2.2.4.5 Sub-cutaneous tumours

Immunohistochemistry:

- Phospho-Smad2 (serine 245,250,255); Slides were dewaxed in xylene and rehydrated with alcohols. Blocking was performed with 3% H_2O_2 in methanol for 10 minutes at ambient temperature followed by a rinsing step in PBS-T twice for 5 minutes. PBS-T was removed and preheated trypsin added for 15 minutes at 37^{o}C. A wash step followed and then sections were blocked using 5% serum/1%BSA/PBS for 30 minutes at ambient temperature. The primary linker pSMAD2 antibody was then incubated overnight in 1%BSA/PBS at 4^{o}C at a concentration of 1:100, excluding negative control. Sections were washed in PBS-T and slides were incubated in biotinylated rabbit secondary antibody 1:200 in 1%BSA/PBS for 30 minutes at ambient temperature. Sections were then washed and incubated in signal enhancer for 30 minutes prior to further washing and incubation with DAB for 3-6 minutes. Sections were then counterstained with haematoxylin, washed and dehydrated with alcohols and xylene before mounting and viewing via light microscopy

- Follistatin; Slides were processed as for phospho-Smad2. The primary follistatin antibody was used at a concentration of 1:200 with biotinylated rabbit secondary antibody at a concentration of 1:200.

2.2.5 Statistical analysis of *in vitro* and *in vivo* data.

All analysis of *in vitro* and *in vivo* data was performed using Graph pad PRISM version 5.0d. Tests chosen for comparisons of groups were based on normality of data, with parametric tests used when data was normally distributed, and non-parametric when normality was not achieved.

2.2.6 Clinical serum samples from the ANZAC and AZURE clinical trials.

2.2.6.1 Ethics and consent

Ethical approval for research on clinical samples, and consent from participants from the ANZAC and AZURE clinical trials is detailed in the relevant chapters (chapters 3 and 4).

2.2.6.2 Trial protocol

ANZAC and AZURE trial protocols are detailed in the relevant chapters (chapters 3 and 4).

2.2.6.3 Clinical serum sample processing

Enzyme linked immune absorbance assay (ELISA) for quantification of protein levels in ANZAC serum samples:

- Human activin, follistatin and TGFβ1 were performed as previously described in section 2.2.2.2
- Human Inhibin A; Inhibin A is an ovarian secreted hormone produced throughout reproductive life, declining to undetectable levels at menopause. It is a member of the TGFβ superfamily of proteins. The inhibin A ELISA was carried out according to manufacturers instructions. Cell culture supernatant was centrifuged at 10,000 rpm for 5 minutes; no dilution of samples was required. Standards were provided ready to use, and ranged from 1000pg/ml to zero. In duplicate, 50μl of standards, control and samples were added to each well that was pre-coated in anti-inhibin A antibody. 50μl of sample buffer A and B was added to each well. The wells were incubated for 3 hours at ambient temperature on an orbital shaker at 500rpms. Wells were washed x6 with wash solution using a squirt bottle, and then 100μl antibody enzyme conjugate was added to each well. The plate was

incubated on the orbital shaker at 500rpm for 1 hour at ambient temperature. Washing steps were repeated and 10μl of TMB chromogen solution added to each well in darkness for 15 minutes on the orbital shaker. Final steps involved addition of 100μl of stopping solution to each well and absorbance read at 450nm using a microplate reader. Inhibin A levels were extrapolated from the standard curve. Minimum detection limits were <5pg/ml, and inra-assay CV's were below 15%.

Hormone radioimmunoassay (RIA's) and electrochemiluminescent immunoassay (ECLs) for AZURE serum samples:

- Human Inhibin A; The Access RIA was used to detect inhibin A (Beckman Coulter). This was a two-step immunoenzymatic assay. Thawed sample/calibrator/quality control was added to a vessel containing paramagnetic particles coupled with anti-inhibin A monoclonal antibody. A wash step removed excess sample and reagents. Anti-inhibin A monoclonal antibody-alkaline phosphatase conjugate was then added to the vessel to detect inhibin A which was bound in the previous reaction. Following incubation, materials bound were held in a magnetic field while unbound substrate was washed away. Chemiluminescent substrate was then added and the quantity of light generated was detected by a luminometer. The amount of light was proportional to the quantity of inhibin A in the sample. The lowest detectable inhibin A level was 1pg/ml. Internal quality control and intra-assay precision data is provided in chapter 3.
- Oestradiol and Follicle Stimulating hormone; ECL was used to detect oestradiol and FSH using the Roche Cobas E602 autoanalyser. Standards or samples are incubated with a biotinylated antibody and a ruthinuim labeled antibody complex to the hormone of interest. Following incubation, streptavidin coated paramagnetic micro particles are added which binds the biotinylated antibody. The subsequent mixtures of immune complexes are magnetically trapped whilst unbound reagent is washed away. The ruthenium conjugate is stimulated with electricity to produce light, the amount of which is proportional to oestradiol or FSH in the sample. Oestradiol detection limit was 18.4 pmol/l, FSH detection

limit was 0.1IU/l. Internal quality control and intra-assay precision is detailed in chapter 3. Excess serum that was not processed was re-frozen for future studies.

2.2.6.4 Statistical analysis of clinical serum data.

Details of the programs used, and analyses performed are detailed in the relevant chapters (chapters 3 and 4).

3. Baseline evaluation of reproductive hormones assists in the selection of postmenopausal patients for adjuvant zoledronic acid.

An AZURE translational study.

3.1 Summary

Several large adjuvant clinical trials, involving thousands of breast cancer patients, have shown that zoledronic acid decreases disease recurrence and increases survival when added to standard therapy in patients who are naturally or chemically postmenopausal. The reproductive hormonal changes occurring in the transition from premenopausal to postmenopausal involve a fall in circulating oestradiol and inhibin A with a concomitant rise in follicle stimulating hormone (FSH).

In early breast cancer, disseminated tumour cells (DTCs) can be detected in the bone marrow, in patients without clinically apparent metastatic disease. The fate of these cells may be affected by reproductive hormones and zoledronic acid, resulting in different outcomes for pre- and postmenopausal women treated with these drugs. Accurately selecting patients for zoledronic acid is important to ensure the population treated derives the most benefit. Baseline serum levels of oestradiol, inhibin A and FSH from 806 patients randomised in the AZURE trial were measured to evaluate if serum hormone levels could be used to predict which patients would benefit from the addition of zoledronic acid to standard adjuvant therapy.

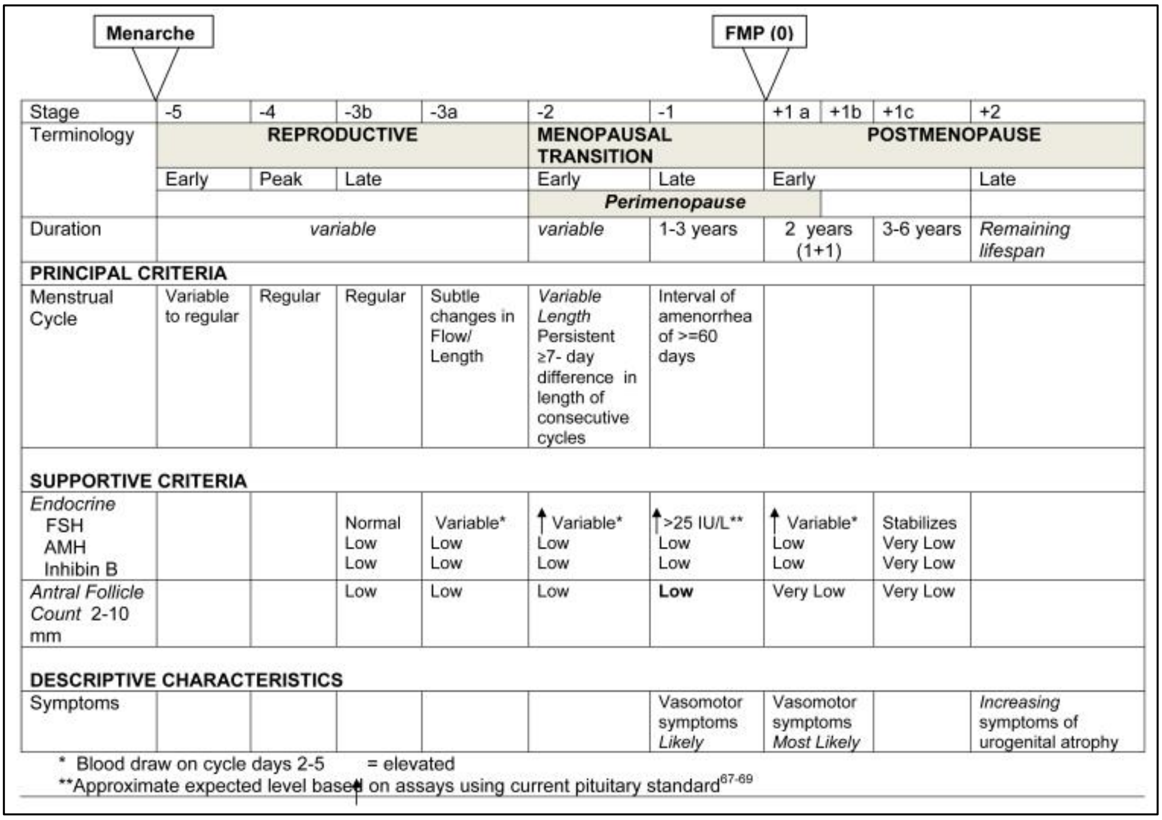
Here we show the baseline characteristics and invasive disease free survival (IDFS) outcomes in the serum population were reflective of the main AZURE population. Clinical and biochemical menopausal status were not concordant in all patients, but in a heterogenous breast cancer population, serum FSH, oestradiol and inhibin A in postmenopausal ranges continued to select patients with improved IDFS outcomes with zoledronic acid. No individual hormone level was driving the interaction with zoledronic acid, indicating the overall activity of the HPG axis influences outcomes with the drug. A low pretreatment FSH (<26IU/l) predicted for a shorter time to skeletal recurrence and a low pretreatment oestradiol (<50pmol/l) predicted for a shorter time to distant recurrence. These results indicate that premenopausal patients may be at increased risk for bone metastases, however postmenopausal patients may have an increased tumor burden in non-skeletal sites. This may reflect the differing effect of reproductive hormones on the bone premetastatic niche, influencing DTC survival in bone or spread to distant sites in early breast cancer.

3.2 Introduction

Bone is a common site for breast cancer metastases (Coleman 2005) and the bone targeted agents, bisphosphonates, have an established role in the prevention of skeletal related events in metastatic disease and management of bone loss associated with cancer therapies (Coleman 2004; Wilson and Coleman 2011). However data are now emerging to show that bisphosphonates improve outcomes in early breast cancer, by modification of the bone microenvironment and reduction in the number of disseminated tumour cells using the bone as a sanctuary for survival (Aft, Perez *et al*. 2012). Three large adjuvant trials have shown improved disease free survival outcomes (DFS) with addition of zoledronic acid in patients with a natural or chemically induced menopause (Coleman, Marshall *et al*. 2011; Gnant, Mlineritsch *et al*. 2011; Coleman, de Boer *et al*. 2013). The largest of these trials was the AZURE trial (does **A**djuvant **Z**oledronic acid red**U**ce **RE**currence in patients with high-risk, localized breast cancer? ISCRCTN 79831382, BIG01/04) and results following 752 DFS events have been published (Coleman, Marshall *et al*. 2011), moreover, an updated report was recently presented after 84 months follow up and 966 DFS events (R. Coleman, R. Burkinshaw *et al*. 2013) showing bone metastases free survival was improved with addition of zoledronic acid in a heterogenous population of pre- and postmenopausal patients (HR 0.81; 95%CI 0.68-0.97, p=0.022). Pre-specified sub-group analyses according to menopausal status continued to show an improvement in invasive DFS (IDFS) (HR 0.77; 95%CI 0.63-0.96) and overall survival (OS) (HR 0.81; 95%CI 0.63-1.04) in postmenopausal patients >5 years since menopause who received zoledronic acid but not any other group (pre-, 1-5 years postmenopausal and unknown). This differential effect of treatment according to menopausal status was driven by a decrease in loco-regional recurrence and extra-skeletal recurrence in patients >5 years postmenopausal receiving zoledronic acid compared to an increase in all other menopausal groups.

The molecular explanation for the differential anti-tumour effect of zoledronic acid according to menopausal status is yet to be identified, but the different reproductive hormone influences in the bone microenvironment could potentially interact with bisphosphonates and affect dormant cancer cells taking sanctuary at this site. The concept of bone acting as a sanctuary for disseminated tumour cells (DTCs) in early breast cancer requires consideration. The presence of DTCs at diagnosis is an independent poor prognostic factor and 50% of patient with detectable DTCs will relapse within 10 years (Braun, Vogl *et al*. 2005). This relapse may be within the bone or at extraosseous sites and can occur decades after the primary tumour diagnosis, suggesting these DTCs may be held in a state of dormancy. Upon activation from unknown triggers, these DTCs develop autonomous growth becoming overt metastases within the bone, or spread via the blood stream to distant sites. These DTCs may have 'stem cell' like properties conferring a resistance to standard chemotherapy, with evidence that primary systemic therapy does not eradicate DTCs, even in patients who have a complete pathological response in the primary tumour (Becker, Solomayer *et al*. 2007). This persistence of DTCs after primary systemic therapy is an independent poor prognostic factor for DFS and OS in the first 5 years following diagnosis (Janni, Vogl *et al*. 2011).

The reproductive hormonal influences in bone will be determined by the relative activity of the hypothalamic pituitary gonadal (HPG) axis. The changes in this axis with increasing age have been defined by the Stages of Reproductive Ageing Workshop (STRAW+10) (Harlow, Gass *et al*. 2012). The female reproductive lifespan was divided into 3 phases; reproductive, menopausal transition and postmenopausal with defined clinical changes in menstrual cycles and measurable changes in reproductive hormones before and after final menstrual period (FMP) (Fig 3.1). With increasing age, FSH demonstrated an increase and stabilized at >25IU/l 3-6 years post FMP, inhibins declined with undetectable levels at the time of FMP, and oestradiol declined but did not stabilize at low levels until up to 8 years post FMP. Clinical menstrual cycle criteria were the principle defining parameters for stage definition in healthy women and biochemical classification was supportive, due to the lack of international standardisation of hormone assays. The impact of these reproductive changes on bone turnover has been investigated. Perrien *et al,* in a cross sectional study of 188 pre- and postmenopausal women aged 21-85 years, correlated oestradiol, FSH, inhibin A and B with bone turnover markers; serum bone alkaline phosphatase (BALP) as a marker of bone formation and C-terminal collagen I cross-link (CTx), urine pyrindinoline and deoxypyrindinoline as markers of bone resorption (Perrien, Achenbach *et al*. 2006). Multivariate analysis of pre- and postmenopausal women showed inhibin A to be a good predictor of markers of bone formation and resorption in premenopausal women, and the best predictor of bone formation markers in postmenopausal women. Oestradiol levels were a better predictor of bone resorption markers in postmenopausal women. FSH was not a significant



Stage	-5	-4	-3b	-3a	-2	-1	+1 a	+1b	+1c	+2
Terminology	**REPRODUCTIVE**				**MENOPAUSAL TRANSITION**		**POSTMENOPAUSE**			
	Early	Peak	Late		Early	Late	Early			Late
					Perimenopause					
Duration	*variable*				*variable*	1-3 years	2 years (1+1)		3-6 years	*Remaining lifespan*
PRINCIPAL CRITERIA										
Menstrual Cycle	Variable to regular	Regular	Regular	Subtle changes in Flow/ Length	*Variable Length* Persistent ≥7- day difference in length of consecutive cycles	Interval of amenorrhea of >=60 days				
SUPPORTIVE CRITERIA										
Endocrine FSH AMH Inhibin B			Normal Low Low	Variable* Low Low	↑ Variable* Low Low	↑>25 IU/L** Low Low	↑ Variable* Low Low		Stabilizes Very Low Very Low	
Antral Follicle Count 2-10 mm			Low	Low	Low	**Low**	Very Low		Very Low	
DESCRIPTIVE CHARACTERISTICS										
Symptoms						Vasomotor symptoms *Likely*	Vasomotor symptoms *Most Likely*			*Increasing* symptoms of urogenital atrophy

* Blood draw on cycle days 2-5 ↑ = elevated
**Approximate expected level based on assays using current pituitary standard[67-69]

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Figure 3.1. The STRAW+10 Staging system for reproductive ageing in women.

Principle defining criteria are based on menstrual cycle changes either side of final menstrual period (FMP). Supportive criteria include endocrine analysis of reproductive hormones before and after FMP.

predictor of any bone turnover markers. Data from *in vitro* and *in vivo* studies have shown that administration of recombinant inhibin A increased bone forming osteoblast activity (Perrien, Akel *et al*. 2007), and blocking the activity of FSH using an antibody to the β subunit increased bone formation parameters and inhibited bone resorption parameters (Zhu, Blair *et al*. 2012). In addition, oestradiol decreases the lifespan of bone resorbing osteoclasts by promoting apoptosis and increases the lifespan of osteoblasts by exerting antiapoptotic effects (Manolagas 2000). Therefore a premenopausal woman will have higher bone mass than a postmenopausal woman in part due to the anabolic effects of high oestradiol, inhibin and concurrently low FSH on osteoblasts, in addition to the suppressive effect of the reproductive hormones on osteoclast function. Expansion of the osteoblastic niche *in vivo* has been shown to increase tumor cell survival (Shiozawa, Pedersen *et al*. 2011), therefore levels of reproductive hormones may affect the ability of this niche to support tumour cell survival in bone.

Based on this evidence, and advice from experts in the field of reproductive research (personal communication from Professor David Robertson, Prince Henry's Institute of Medical Research, Victoria, Australia, and Professor Dana Gaddy, University of Arkansas, Texas), FSH, oestradiol and inhibin A were identified as being of importance in biochemically defining menopausal status and having relevant effects on the bone microenvironment which potentially may interact with zoledronic acid in breast cancer.

3.3 Aims

The overall purpose of this chapter was to assess if baseline serum FSH, oestradiol and inhibin A from patients recruited to the AZURE trial could be used to select patients for adjuvant zoledronic acid, and if baseline hormones were either prognostic for breast cancer outcomes or were predictive of a treatment benefit with zoledronic acid. The primary endpoint of the AZURE trial was disease-free survival (DFS) with secondary endpoints including overall survival and invasive disease free survival (IDFS). For this analysis the focus was IDFS of the following types; all IDFS, skeletal only IDFS and non-skeletal distant IDFS.

The following aims were set;

1. Compare baseline demographics of the AZURE serum population (n=872) to the overall AZURE population (n=3360).

2. Evaluate if biochemical classification of menopausal status using FSH, oestradiol and inhibin A concurs with patient reported clinical classification.

3. Assess if biochemical classification of menopause using FSH, oestradiol and inhibin A predicts IDFS outcomes with zoledronic acid.

4. Assess if baseline individual hormone levels can predict IDFS outcomes with zoledronic acid.

5. Evaluate the prognostic value of baseline hormones levels for bone only distant recurrence and any distant recurrence as first event.

3.4 Patients and Methods

3.4.1 Patient inclusion and exclusion criteria.

The AZURE trial was an academic, multi-centre, international phase III trial that recruited 3360 women with node positive Stage II/III breast cancer. Patients were randomized 1:1 to receive standard adjuvant therapy (chemotherapy and/or endocrine therapy) +/- the bone-targeted drug, zoledronic acid, 4mg IV for 5 years, initially q3-4 wk, then with increasing intervals between doses (Chapter 1, Fig 1.7). Patients were randomized using a computer generated system which included the following minimization criteria; number of involved lymph nodes, clinical tumour stage, oestrogen receptor status, menopausal status, type and timing of systemic therapy, study center and statin use. The primary endpoint was DFS with pre-specified subgroup analyses planned for the minimization criteria; secondary endpoints included OS and IDFS. Investigations for confirmation of recurrence events were as deemed appropriate by the treating physician. The date of recurrence defined as the date on which relapse was first suspected to minimize ascertainment bias and validated by either on site or telephone based monitoring. All data were recorded on the central database at Leeds CTRU.

Eligibility criteria for the AZURE trial included patients aged >18 years with histologically confirmed breast cancer with N1 disease or T3-4 primary tumour. Patients were excluded if there was clinical or imaging evidence of metastases or if the primary tumour could not be fully surgically excised. Baseline serum samples were available from 872 patients recruited into the AZURE trial. The serum was stored at -80^{o}C at Cancer Clinical Trials Unit, Sheffield, UK and was collected according to the AZURE laboratory manual, version 1, 30th April 2004 (see appendix 1). For inclusion in the serum hormone analyses, patients had to have confirmed written consent to storage and future analysis of serum. If the above criteria were not fulfilled then patients were excluded. In addition, 60 patients were excluded because they were taking medication (hormone replacement therapy, tibilone and tamoxifen) at the time of serum collection that may alter levels of reproductive hormones. Initially, neo-adjuvant patients were also excluded from the analysis over concerns that tumour secretion of inhibin A may affect results. However, after review of the inhibin A levels, the decision was made to include these patients since the levels were reflective of the patients clinical menopausal status and generally within the expected range. In total, 806 patients were eligible for inclusion in the hormone analysis.

3.4.2 Biochemical definition of postmenopausal.

Using assay specific reference ranges for pre- and postmenopausal, criteria were set for fulfillment of an established biochemical postmenopausal status. These criteria were stringent and chosen to minimize the cross over with premenopausal nadir serum levels during the menstrual cycle. To fulfill a definition of biochemically ‘postmenopausal’ the following had to be met;

1. FSH ≥ 26IU/l
2. Oestradiol ≤ 50pmol/l
3. Inhibin A ≤ 3.6pg/ml

If any one or more of these criteria were not fulfilled the patient was classified as non-postmenopausal..

3.4.3 Hormone evaluation.

Inhibin A analysis was performed on the automated ACCESS chemiluminescence immunoassay system from Beckman Coulter Ref A36097. Oestradiol and FSH analysis were performed on the automated Roche 602 Elecsys electrochemiluminescence immunoassay. FSH levels were standardized to the second International Reference Preparation 78/549. Lower limit of detection for the assays were as follows; inhibin A <1pg/ml, oestradiol 18.4pmol/l, FSH 0.1 IU/L. Internal quality control materials were run every 24 hours covering 3 levels of analyte (low, medium and high), and reference ranges for pre- and postmenopausal women were assay specific, determined by the manufacturer and validated in-house (Table 3.1). All laboratories used for analysis were Clinical Pathology Accredited, ensuring strict operational guidelines and standards within Sheffield Teaching Hospitals (STH) NHS Trust, UK. The planned analyses were registered with the STH Research Co-ordinator, with agreement from lab managers regarding movement of samples across the city to minimize freeze thaw cycles.

Table 3.1 Internal quality control data for inhibin A, oestradiol and FSH including assay dependent reference ranges for postmenopausal and premenopausal.

Hormone	Average monthly internal QC data			Reference ranges
Inhibin A (pg/ml)	**Low**	**Med**	**High**	
Mean	159.1	438.8	869.2	Premenopausal (cycle dependent) 5-160 pg/ml
SD	7.5	20.4	39.8	
CV%	4.7	4.6	4.6	Postmenopausal 0-3.6pg/ml
Oestradiol (pmol/l)	**Low**	**Med**	**High**	
Mean	170	1059	2576	Premenopausal (cycle dependent) 46-1828pmol/l)
SD	11.4	25.2	54.5	
CV%	6.7	2.3	2.1	Postmenopausal 18.4-201pmol/l
FSH (IU/l)	**Low**	**Med**	**High**	
Mean	5.5	18.1	39.08	Premenopausal (cycle dependent) 1.7-22 IU/l)
SD	0.09	0.23	0.53	
CV%	1.7	1.29	1.36	Postmenopausal 26-135IU/l

Internal quality control material was run every 24 hours covering 3 levels of analyte (low, medium and high). All laboratories were CPA accredited. The definitions of 'biochemically postmenopausal' were based on the assay specific cut points for FSH and inhibin A. An oestradiol of <50pmol/l was chosen to define 'biochemically postmenopausal' to avoid the nadirs in cycling serum oestradiol in premenopausal women

3.4.4 Data collection from central database.

Demographic, treatment and outcome data for all patients recruited to AZURE were held on a password protected 'Makro' database at the Clinical Trials Research Unit (CTRU), Leeds. Access to this database was limited to the senior AZURE trial statistician, Helen Marshall and medical statistician Samantha Hinsley. The following data were extracted;

1. Patient characteristics at randomisation;

- Trial number
- Age
- Date of randomisation
- Menopausal status; Premenopausal, <5 years since menopause, unknown menopausal status, >5 years since menopause.

2. Disease characteristics at randomisation

- ER status; Positive, negative, unknown.
- Tumour stage at diagnosis; T1-4.
- Number of lymph nodes involved; 0, 1-3, >4, unknown.

3. Treatment characteristics:

- Randomised to; Interventional arm (zoledronic acid) or control arm (no zoledronic acid)
- Systemic therapy; Chemotherapy, endocrine, chemotherapy+endocrine.
- Timing of chemotherapy; Adjuvant/neo-adjuvant

4. Disease outcomes

- Invasive disease free survival (IDFS); subdivided into skeletal distant recurrence, non-skeletal distant recurrence, loco-regional recurrence, second malignancy, IDFS minus skeletal recurrence, all IDFS.

3.4.5 Study approval for translational serum analysis

This translational study was approved by the AZURE Trial Steering Committee (TSC) and Data Monitoring Committee (DMEC) in August 2011.

3.4.6 Statistical methodology

Statistical analysis was carried out by Helen Marshall and Samantha Hinsey at the Clinical Trials Unit, Leeds. 966 disease free survival events were observed in the AZURE trial. Statistical analysis was carried out after a median follow-up of approximately 84 (IQR 71-92) months for patients included in the endocrine analyses. Cut points used for analyses of hormones (high versus low) were as previously described for non-postmenopausal versus postmenopausal categorization. Kaplan-Meier survival curves were used to evaluate rates of invasive disease free survival with differences between groups assessed using the log-rank test and Cox's proportional hazards model to adjust for tumour stage, ER status, lymph node involvement and neo-adjuvant therapy as per the main AZURE subgroup analyses. Time to first distant recurrence analyses were adjusted for tumour stage, ER status and lymph node status. Time to bone as first recurrence was adjusted for randomised treatment allocation, tumour stage and lymph node involvement as per the main AZURE subgroup analyses. Analyses were also adjusted for randomised treatment allocation when assessing the interaction of individual hormone levels with treatment. P-values were considered significant at α <0.05. All analyses were performed with the use of SAS software version 9.2.

3.4.7 Funding and publication

The cost of collection of the serum samples was provided by the research infrastructure support at participating sites in the UK by the National Cancer Research Network. The cost of shipping and analyses of FSH, oestradiol and inhibin A were provided by a grant award from Novartis Pharmaceuticals. Part of these data were presented as an oral presentation at San Antonio Breast Cancer Symposium, Texas, December 2012 based on the 2010 data lock used for the interim analysis. These analyses have been subsequently updated and use the 2013 datalock.

3.5 Results

3.5.1 Demographics and baseline characteristics of serum population compared to overall study population.

Baseline data were available for the 872 patients with stored serum. The serum population was reflective of the overall AZURE study population with respect to lymph node involvement, T stage, ER status and menopausal status (Table 3.2). Within the 872 patient serum population, the treatment groups (zoledronic acid and control) were well matched (Table 3.3). 60 patients at baseline were established on hormone replacement therapy, endocrine therapy or tibilone and excluded from further hormone analysis.

IDFS outcomes in the serum population were comparable to the main AZURE outcomes and showed a similar heterogeneity of treatment effect according to menopausal status, with postmenopausal patients having improved IDFS with zoledronic acid, an effect not seen in non-postmenopausal patients (postmenopausal HR for zoledronic acid vs control; main AZURE population 0.77; 95%CI 0.63-0.96 and serum population 0.728; 95% CI 0.470-1.129) (Fig 3.2). The differing effect of zoledronic acid between menopausal subgroups, although significant in the overall AZURE population (p=0.030), was not significant in the serum population (p=0.3148), which may be due to the smaller sample size.

These data confirmed the serum population was a true representation of the overall population.

3.5.2 Biochemical classification of menopausal status versus clinical classification.

Median hormone levels (range) in the clinically pre- and postmenopausal patients showed the expected fall in oestradiol from 392pmol/l (19-4278) in premenopausal patients to 55.8 pmol/l (19-934) in postmenopausal patients, a fall in inhibin A from 22.7pg/ml (0.2-170) in premenopausal patients to 1.6pg/ml (0.1-38.9) in postmenopausal patients and a rise in FSH from 18.5IU/l (1.1-133) in premenopausal patients to 71 IU/l (3.5-194) in postmenopausal patients. There was not complete agreement between clinical

Table 3.2 Baseline characteristics of the overall AZURE population and the serum AZURE population

	Overall Study (n = 3360)	Serum samples (n = 872)
Lymph nodes		
0	61 (1.8%)	16 (1.8%)
1-3	2073 (61.7%)	534 (61.0%)
>4	1212 (36.0%)	320 (36.6%)
Unknown	13 (0.4%)	2 (0.4%)
T stage		
T1	1065 (31.7%)	285 (32.6%)
T2	1718 (51.1%)	427 (48.8%)
T3	455 (13.6%)	131 (15.0%)
T4	117 (3.5%)	29 (3.4%)
ER status		
Positive	2635 (78.4%)	676 (77.2%)
Negative	704 (21.0%)	192 (22.0%)
Unknown	20 (0.6%)	4 (0.5%)
Menopausal status		
Premenopausal	1503 (44.8%)	409 (46.8%)
≤ 5 years post	491 (14.6%)	123 (14.2%)
> 5 years post	1041 (31.0%)	266 (30.5%)
Status unknown	324 (9.6%)	74 (8.6%)

The serum population was similar to the overall main study population in terms of lymph nodes, T stage, ER status and menopausal status

Table 3.3. Baseline characteristics for the serum AZURE population according to treatment received.

	Standard therapy (n=434) N (%)	Standard therapy+Zoledronic acid (n=431) N (%)
Tumour stage		
T1	136 (31.3)	147 (34.1)
T2	225 (51.8)	200 (46.4)
T3	57 (13.1)	71 (16.5)
T4	16 (3.7)	13 (3.0)
Neo-adjuvant therapy		
Yes	23 (5.3)	28 (6.5)
No	411 (94.7)	403 (93.5)
Systemic therapy		
Endocrine alone	20 (4.6)	13 (3.0)
Chemotherapy alone	93 (21.4)	95 (22.0)
Endocrine and chemotherapy	321 (74)	323 (74.9)
Anthracycline		
Yes	402 (92.6)	410 (95.1)
No	32 (7.4)	21 (4.9)
Taxane		
Yes	85 (19.6)	91 (21.1)
No	349 (80.4)	340 (78.9)
Menopausal status		
Premenopausal	205 (47.2)	199 (46.2)
<5 years since menopause	63 (14.5)	61 (14.2)
>5 years since menopause	131 (30.2)	132 (30.6)
Unknown menopausal status	35 (8.1)	39 (9.0)
ER status		
Positive	333(76.7)	338 (78.4)
Negative	99 (22.8)	91 (21.1)
Unknown	2 (0.5)	2 (0.5)
Lymph node involvement		
0	7 (1.6)	9 (2.1)
1-3 nodes	271 (62.4)	258 (59.9)
≥4	156 (35.9)	162 (37.6)
Unknown	0 (0)	2 (0.5)
Histological grade		
1	34 (7.8)	32 (7.4)
2	175 (40.3)	184 (42.7)
3	219 (50.5)	204 (47.3)
Hormone replacement therapy at baseline		
Yes		
No	5 (1.2)	4 (0.9)
	429 (98.8)	426 (98.8)
Tibilone at baseline		
Yes	0 (0)	1 (0.2)
No	434 (100)	430(99.8)
Endocrine therapy at baseline		
Yes	29 (6.7)	21 (4.9)
No	405 (93.3)	410 (95.1)

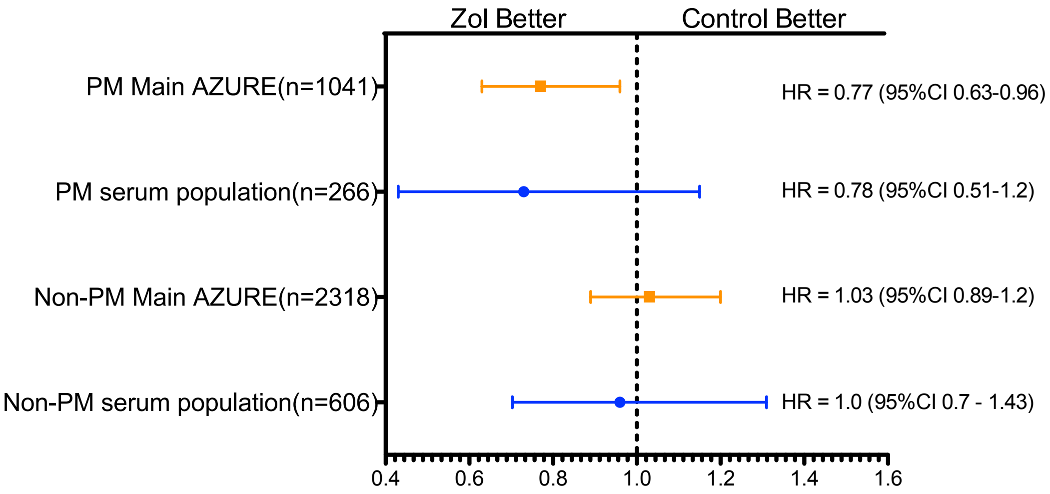


Figure 3.2 Invasive disease free survival (IDFS) outcomes in the main AZURE population and the serum AZURE population.

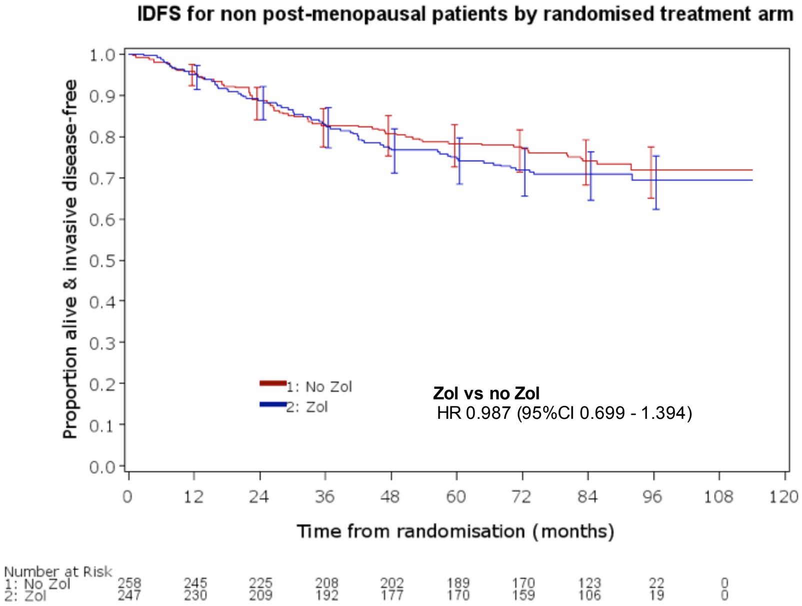
Data represents adjusted hazard ratios (HR) and 95% confidence interval (CI) for the main AZURE population and the serum AZURE population according to menopausal sub-groups; postmenopausal (PM) and non-postmenopausal (non-PM). The heterogeneity of treatment effect according to menopausal status was significant in the main AZURE population (p=0.030) but not in the serum AZURE population (p=0.315).

menopausal status and biochemical menopausal status (defined in section 3.4.2). Thirty one (7.4%) clinically premenopausal women were biochemically classified as postmenopausal, of these 1 woman was aged 29 and the remainder were aged ≥44 years. Forty three (15.9%) clinically > 5 years postmenopausal women were biochemically classified as non-postmenopausal. Of these 43 women, the biochemical classification of postmenopausal was not attained due to an oestradiol >50pmol/l in 31 women (72%), an FSH <26IU/l in 11 women (25%) and an inhibin A >3.6pg/ml in 11 women (25%).

These data show that a biochemical definition of postmenopausal status is not 100% concordant with clinical menopausal status. In clinically > 5 years postmenopausal patients this appears to be driven by oestradiol levels that remain higher than the postmenopausal cut point in over half of the discordant patients. In clinically premenopausal patients, only 1 patient was aged <44 years, suggesting that older premenopausal patients may have reproductive profiles that are within biochemically postmenopausal ranges despite regular menses.

3.5.3 Invasive disease free survival according to treatment allocation in patients with a biochemically defined menopausal status.

Among the patients biochemically classified as postmenopausal using FSH, oestradiol and inhibin A, IDFS was improved with addition of zoledronic acid compared to standard therapy (HR 0.809; 95% CI 0.537-1.220), although the confidence intervals overlap unity due to relatively small number of events. However, biochemically non-postmenopausal women had similar IDFS irrespective of treatment allocation (HR 0.987; 95% CI 0.699-1.394) (Fig 3.3). Unlike in the main analysis of all patients, this interaction between treatment and biochemical menopausal status was not statistically significant (p=0.4699), likely due to smaller numbers in the serum population. Considering skeletal IDFS, a trend for benefit of treatment with zoledronic acid was seen in both biochemically postmenopausal and non-postmenopausal patients (postmenopausal HR;0.813, 95%CI 0.367-1.799, non-postmenopausal HR; 0.822, 95%CI 0.463-1.460), however, non-skeletal distant IDFS showed differing affects with addition of zoledronic acid according to menopausal status with a trend to benefit in biochemically postmenopausal patients



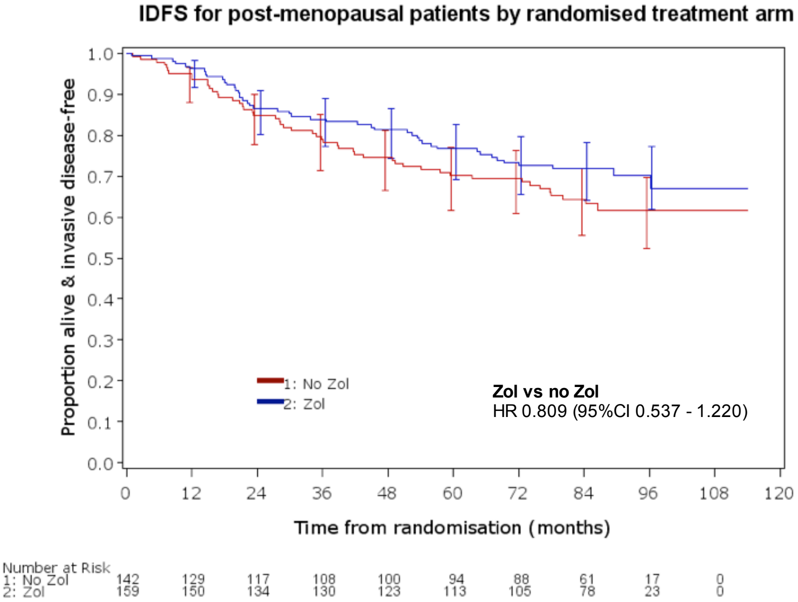


Figure 3.3 Invasive disease free survival according to biochemical definition of menopausal status in the serum AZURE population.

IDFS in biochemically non-postmenopausal patients (top) and biochemically postmenopausal patients (bottom). Menopause: treatment interaction p value 0.469

(postmenopausal HR 0.905; 95%CI 0.494-1.655), and potential increase in non-skeletal distant recurrence in non-postmenopausal patients (HR 1.558;95%CI 0.895-2.713). These treatment menopause interactions were not statistically significant due to wide confidence intervals, but the hazard ratios mirrored the results of the overall AZURE population. Among the patients clinically classified as <5 years post menopause (n=124), 70 (56.4%) were biochemically non-postmenopausal and 54 (43.5%) were biochemically postmenopausal. Of the patients with unknown menopausal patients (n=74), 27 (36.5%) were biochemically non-postmenopausal and 47 (63.5%) were biochemically postmenopausal. The clinical classification of menopause in these patients is less discernable than in menstruating premenopausal women and women many years post menopausal, and therefore IDFS outcomes were evaluated using biochemical menopausal status in these patients in addition to clinical menopausal status for all other patients. IDFS was improved in postmenopausal patients (HR 0.806; 95%CI 0.657-0.990) with addition of zoledronic acid, but not in non-postmenopausal patients (HR 1.043; 95%CI 0.873-1.246) interaction p value 0.064 (Fig 3.4). This heterogeneity of treatment effect according to menopausal status was again driven by a decrease in non-skeletal distant recurrence in postmenopausal women with an increase in non-postmenopausal women (HR postmenopausal 0.878;95%CI 0.644-1.195 vs HR non-postmenopausal 1.271;95% CI 0.966-1.673 p=0.0795), rather than differences in skeletal recurrence.

These data suggest that biochemical classification of a heterogeneous population of breast cancer patients into postmenopausal and non-postmenopausal groups using FSH, oestradiol and inhibin A continues to predicts a trend to improved IDFS in biochemically postmenopausal patients with addition of zoledronic acid, driven by a decrease in non-skeletal distant recurrences. Moreover, using biochemical classification of menopause for women in whom clinical self reporting may be inaccurate (i.e <5 years since menopause or unknown menopausal status) in conjunction with clinical classification for premenopausal and established (>5 years) postmenopausal continues to show a trend for improved IDFS in postmenopausal patients.

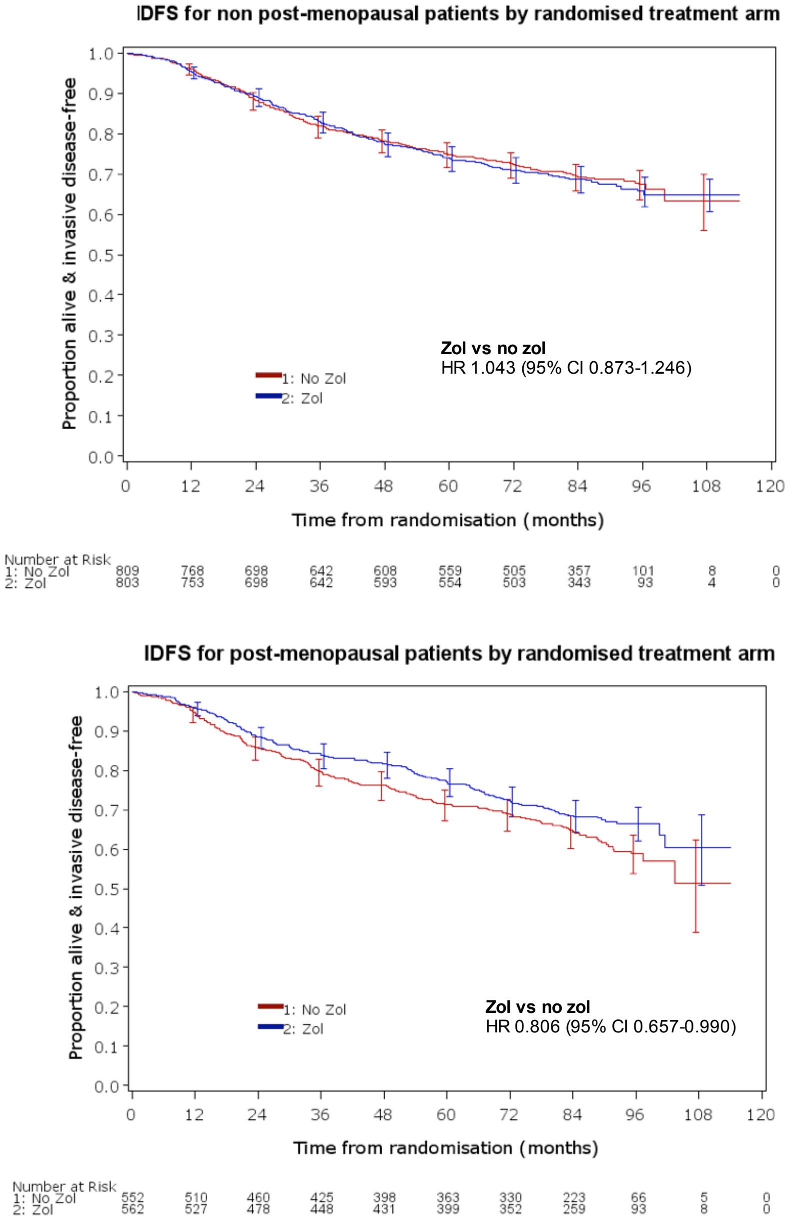


Figure 3.4 Invasive disease free survival using a biochemical definition of postmenopausal or non postmenopausal for women <5 years postmenopausal or unknown and a clinical definition for all others.

IDFS in non-postmenopausal patients (top) and postmenopausal patients (bottom). Menopause: treatment interaction p value 0.064.

3.5.4 Predictive value of non-postmenopausal versus postmenopausal levels of FSH, oestradiol or inhibin A for IDFS outcomes with zoledronic acid.

IDFS was similar with addition of zoledronic acid in patients with a high or low FSH (>26IU/l; HR 0.932 95%CI 0.657-1.321, ≤26IU/l; HR 0.872 95%CI 0.538-1.305). However in patients with baseline FSH ≤26IU/l, there was a trend to a decreased skeletal distant recurrence with zoledronic acid (HR 0.713 95%CI 0.381-1.334) but an increased non-skeletal distant recurrence (HR 1.314 95%CI 0.62-2.493). This was mirrored in the IDFS outcomes with inhibin A and oestradiol, which negatively regulate FSH secretion; inhibin ≥3.6pg/ml showed a trend for an improved skeletal IDFS with zoledronic acid (HR 0.596 95%CI 0.3-1.185) but a worse non-skeletal IDFS (HR 1.504 95%CI 0.787-2.874), oestradiol ≥50pmol/l showed a trend for an improved skeletal IDFS with zoledronic acid (HR 0.78 95%CI 0.430-1.416) but a worse non-skeletal IDFS (1.505 95%CI 0.85-2.664)(Table 3.4).

These data suggest no single hormone is predictive of an improvement in IDFS in bone or outside of bone with addition of zoledronic acid, but reproductive hormone levels in the non-postmenopausal range (low FSH, high inhibin and high oestradiol) continue to predict a worse extraskeletal IDFS with zoledronic acid as per the results of the main AZURE trial.

3.5.5 Prognostic value of pre- versus postmenopausal levels of FSH, oestradiol or inhibin A on bone recurrence and distant recurrence.

An FSH ≤26IU/l (non-postmenopausal) was borderline significant for a shorter time to bone as first recurrence compared to an FSH >26IU/l (HR 0.66 95%CI 0.41-1.04 p=0.0721)(Fig 3.5). Inhibin A or oestradiol were not significantly associated with time to bone recurrence. An oestradiol <50pmol/l (postmenopausal) was borderline significant for a shorter time to distant recurrence (bone+non bone) compared to an oestradiol ≥50pmol/l (HR 1.33 95%CI 0.98-1.82 p=0.0646) (Fig 3.6). Considering there was no significant difference in time to bone as first recurrence according to oestradiol levels, this effect of low oestradiol on a shorter time to distant recurrence may be driven by an increase in metastatic disease recurring outside of bone. Inhibin A or FSH were not significantly associated with time to distant recurrence.

Table 3.4 IDFS outcomes for zoledronic acid vs control in patients with high versus low serum levels of reproductive hormones.

Outcomes are separated in to all IDFS events, skeletal distant recurrence and non-skeletal distant recurrence. Hazard ratio (HR) >1 indicates risk of experiencing an event is greater in the zoledronic acid arm, HR <1 indicated risk of experiencing an event is greater in the control arm.

Hormone	All IDFS HR (95%CI)	Skeletal distant recurrence HR (95%CI)	Non-skeletal distant recurrence HR (95%CI)
FSH IU/l			
>26	0.932 (0.657-1.321)	1.008 (0.501-2.030)	1.170 (0.695-1.971)
≤26	0.872 (0.583-1.305)	0.713 (0.381-1.334)	1.314 (0.62-2.493)
Inhibin A pg/ml			
<3.6	0.943 (0.671-1.326)	1.086 (0.573-2.058)	1.080 (0.643-1.815)
≥3.6	0.862 (0.571-1.302)	0.596 (0.3-1.185)	1.504 (0.787-2.874)
Oestradiol pmol/l			
<50	0.801 (0.542-1.184)	0.881 (0.416-1.866)	0.976 (0.548-1.738)
≥50	0.993 (0.694-1.421)	0.781 (0.430-1.416)	1.505 (0.850-2.664)

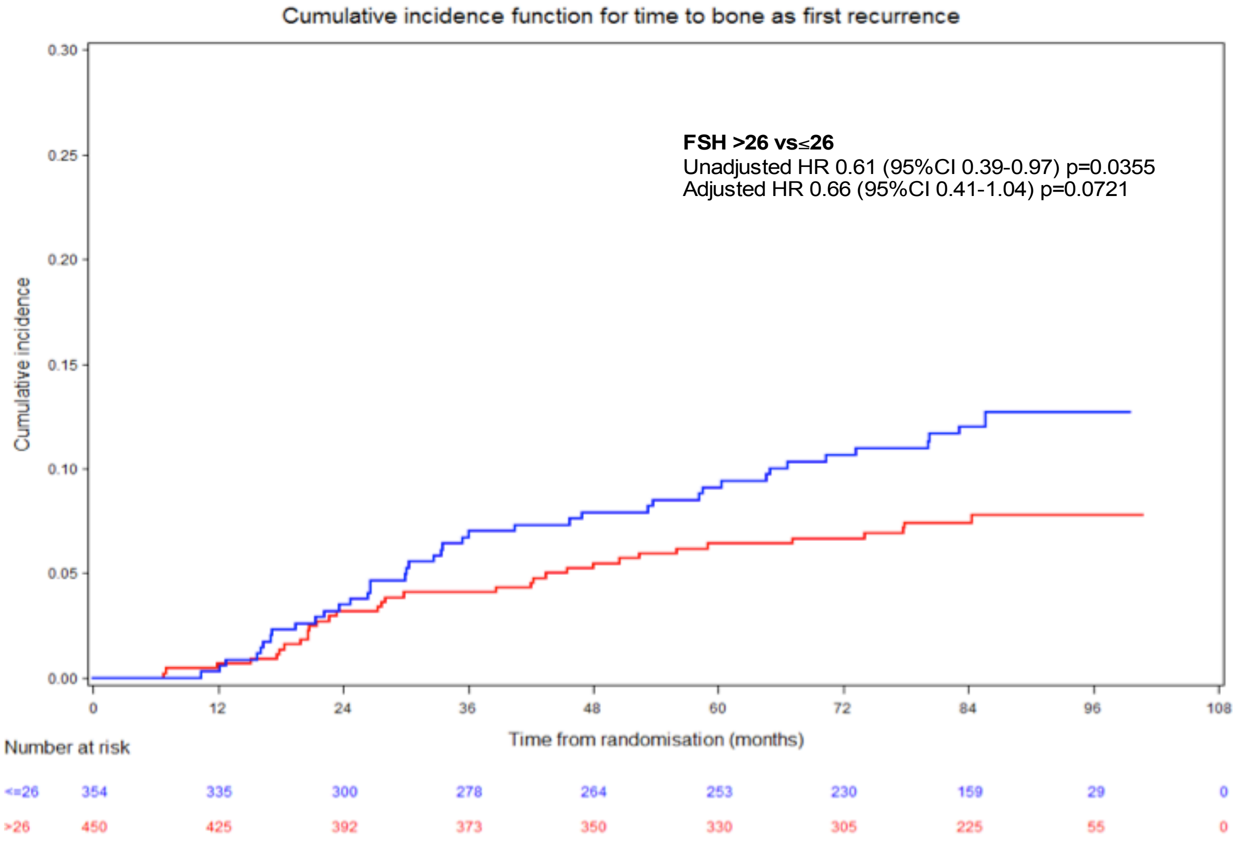
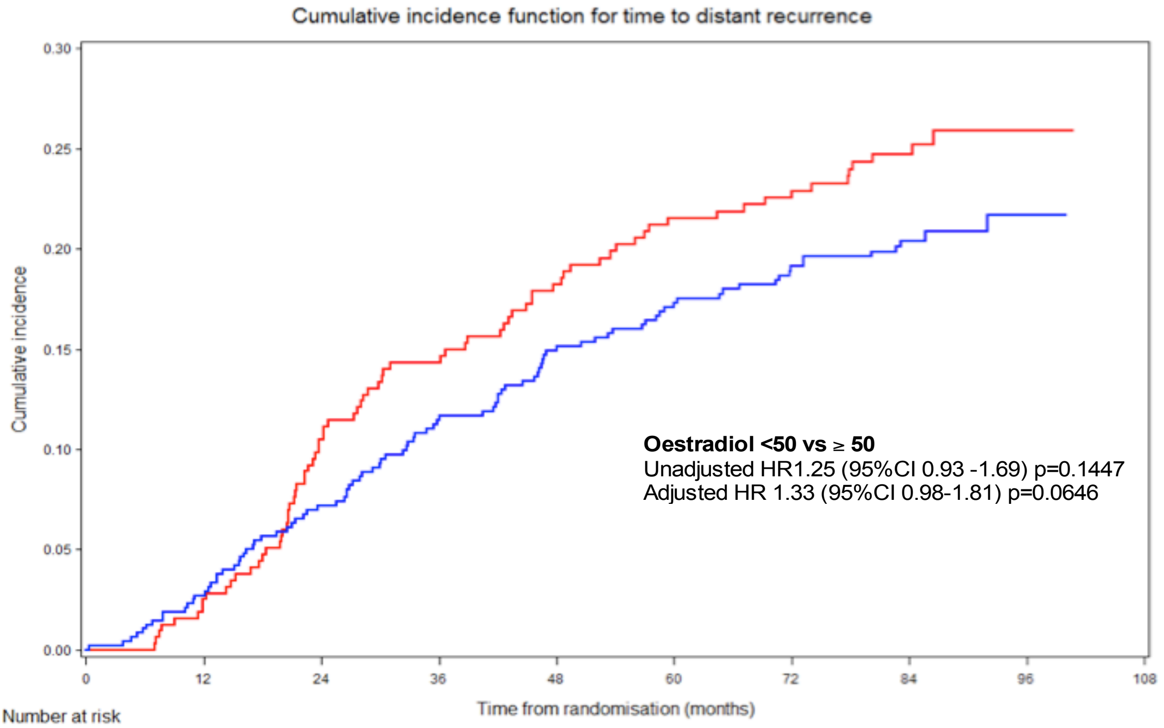


Figure 3.5 Baseline FSH as a prognostic marker for bone recurrence.

Time to bone as first recurrence in patients with serum FSH <26IU/l (blue line) vs >26IU/l (red line).



Number at risk

=>50	482	459	428	401	381	361	330	231	41	0
<50	322	306	275	261	241	227	208	155	44	0

Figure 3.6 Baseline oestradiol as a prognostic marker for distant recurrence.

Time to distant recurrence as first event in patients with serum oestradiol <50pmol/l (red line) vs ≥50pmol/l (blue line)

3.5 Discussion

These data show that clinical and biochemical classification of menopause do not always concur. However, in a heterogenous population of breast cancer patients biochemically classifying patients as postmenopausal using FSH, oestradiol and inhibin A, predicted the same trend of an improved IDFS with zoledronic acid as was shown in the main AZURE trial in patients who were clinically >5 years postmenopausal. No single hormone was predictive of a response to zoledronic acid, suggesting that either a combination of hormones or the overall 'activity' of the HPG axis influences the anti-tumour efficacy of zoledronic acid. In addition, low pretreatment FSH and oestradiol may provide prognostic information on the risk of both bone and distant recurrence respectively.

The disparity between clinical and biochemical classification of menopause in the AZURE breast cancer population was not unique to this population and has been demonstrated in healthy populations. Although longitudinal studies in healthy populations have characterized age specific mean hormone levels, the extrapolation of this to an individual is more challenging. It is generally accepted that a single measurement of FSH or oestradiol in an individual may not a reliable way to stage menopause (Burger, Hale *et al*. 2008), given that ovarian function can fluctuate over time. This is especially of relevance in women in the years around final menstrual period (FMP), when hormonal measurements of FSH and oestradiol are unpredictable and variable (Burger 2011). AZURE did not collect serial serum samples and therefore only the baseline sample could be used to evaluate levels of FSH, oestradiol and inhibin A. In the clinically premenopausal patients who appeared to be biochemically postmenopausal (n=31), only 1 patient was > 44 years old. Older premenopausal women have been shown to have an increasing frequency of anovulatory menstrual cycles compared to younger menstruating women (Hale, Zhao *et al*. 2007; Burger, Hale *et al*. 2008). These anovulatory cycles have differing hormone profiles compared to ovulatory cycles and were biochemically characterized in a longitudinal study of menstruating women aged 45-47 (n=13) over 4-9 years. As the women approached their FMP the menstrual cycles showed an increase in FSH and concurrently low inhibin and oestradiol (Landgren, Collins *et al*. 2004), a biochemical profile which is more consistent with a postmenopausal status. These data therefore suggest that the older menstruating patients in the AZURE trial may have had biochemical hormone profiles that were more in keeping with a postmenopausal status.

In the patients who were clinically >5 years postmenopausal, 43 (15.9%) had a biochemical profile that appeared non-postmenopausal due to any one of FSH, oestradiol or inhibin A not being within postmenopausal ranges. The majority of this group of patients failed to attain a biochemical classification of postmenopausal due to an oestradiol level >50 pmol/l (70%), rather than an inhibin A or FSH out of postmenopausal ranges. This persistently elevated serum oestradiol in women many years postmenopausal has several potential explanations. Firstly, the reproducibility of self reported age at natural menopause has been shown to diminish with increasing time since menopause (Colditz, Stampfer *et al*. 1987), and therefore inaccurate patient recall of the date of natural menopause may have resulted in some women <5 years since menopause self reporting a natural menopause >5 years previous. Secondly, the rate of change in individual serum hormone levels during the years around FMP are not analogous. Data from a longitudinal study (n=150) showed FSH increased 2 years prior to FMP with mean serum levels being within postmenopausal range at the time of FMP. Inhibins begun to decline several years prior to FMP and were undetectable in 50% of women at the time of FMP. However, oestradiol begun to fall at 1 year prior to FMP, but serum levels did not reach low stable nadirs until +2 to +5 years post FMP (Burger, Dudley *et al*. 1999), thus women up to +5 years post FMP may still have oestradiol levels that are higher than the cut point used in the AZURE trial for defining postmenopausal (<50pmol/l). Thirdly, a raised body mass index (BMI) in postmenopausal women is associated with increased serum oestrogen (Folkerd and Dowsett 2013), due to elevated fat conversion of androgens to oestone via the enzyme aromatase (Nelson and Bulun 2001). The median (range) BMI in the AZURE patients >5 years postmenopausal with oestradiol levels ≥50pmol/l was higher (32.32; 22.76-53.58) than those with levels <50pmol/l (26.92; 19.22-47.8), indicating that a raised BMI in the patients >5 years postmenopausal may have contributed to an elevated oestradiol resulting in inaccurate biochemical classification of non-postmenopausal.

These data suggest that a single biochemical evaluation of FSH, oestradiol and inhibin A should not solely be used to select postmenopausal patients for adjuvant zoledronic acid, but should be interpreted with consideration of patient reported clinical menopausal status.

However, despite the discrepancies between clinical and biochemical classification of menopausal status, when the serum AZURE population were solely biochemically classified as postmenopausal or non-postmenopausal irrespective of clinical classification, patients with a biochemical postmenopausal status continued to derive benefit from zoledronic acid. The hazard ratios indicated a trend to an improved IDFS driven by a reduction in non-skeletal disease recurrence in biochemically postmenopausal patients, with the opposite trend in IDFS outcomes in biochemically non-postmenopausal patients. Therefore selecting patients for adjuvant zoledronic acid may be assisted by baseline evaluation of reproductive hormones to ensure that the population receiving the drug gains benefit, and patients who may experience a detrimental effect in terms of increased extra-skeletal recurrence are not exposed.

The selection of patients for adjuvant zoledronic acid using a combination of both self reported clinical menopausal status and complementary biochemical evaluation of baseline reproductive hormones is not possible in patients with unknown clinical menopausal status for reasons such as a previous hysterectomy. Using only biochemical serum levels of reproductive hormones to select premenopausal breast cancer patients with an unknown menopausal status for adjuvant goserelin+/- zoledronic acid has already been undertaken in the prospective randomized ABCSG-12 trial. Eligibility criteria for trial entry included a menstrual bleed within the previous 12 months, and in patients who were of an unknown reproductive status, FSH and oestradiol at baseline were measured to confirm premenopausal ranges prior to initiation of goserelin + endocrine therapy+/- zoledronic acid (Gnant, Mlineritsch *et al.* 2011). As discussed previously (section 1.3.1), zoledronic acid improved DFS in this trial. In the AZURE serum population, a biochemical classification of patients with unknown menopausal status or <5 years since menopause, in conjunction with self reported clinical menopausal status for all other patients continued to show the same trend for a reduction in invasive disease recurrence with addition of zoledronic acid in postmenopausal patients. These data suggested that biochemical evaluation alone may be able to select patients for adjuvant zoledronic acid with a clinically unknown menopausal status or within 5 years since menopause. However the number of IDFS events in this group was small (n=44) and therefore the overall results were dominated by the IDFS outcomes from the clinically classified patients. Longitudinal data evaluating the dynamic changes in serum hormones in the years either side of the final menstrual period have shown that it is not until +5 years post

FMP that hormone levels stabilise (Burger, Dudley *et al.* 2002), suggesting that serial measurements of reproductive hormones would likely be needed when using biochemical classification alone in selecting this subgroup of patients for adjuvant zoledronic acid.

The results presented so far suggested that a reproductive hormone profile consisting of FSH, oestradiol and inhibin A could select patients with improved IDFS outcomes with zoledronic acid. Further evaluation of high versus low levels of each individual hormone as a predictor of response to zoledronic acid showed that no single hormone was affecting the anti-tumour efficacy of zoledronic acid. Therefore, it appears to be the overall activity of the HPG axis that is influencing the efficacy of zoledronic acid. Consideration of the different reproductive profiles from the AZURE trial compared to the ABCSG-12 trial indicate that oestradiol and/or inhibin A may be more important in influencing outcomes with adjuvant zoledronic acid than FSH. In the ABCSG-12 trial all patients received goserelin, with chemically induced low levels of FSH, oestradiol and inhibins, and demonstrated an improvement in DFS both in bone and outside of bone with zoledronic acid (Gnant, Mlineritsch *et al.* 2011). In AZURE, the patient population that derived improvements in DFS and OS were >5 years postmenopausal and therefore had high FSH levels in addition to low oestradiol and inhibins. These data suggest that the ovarian hormones oestradiol and/or inhibin A may be more important in affecting the anti-tumour efficacy of zoledronic acid. This is in part supported by a pre planned subset analysis of ABCSG-12 by patient age at study entry. Zoledronic acid did not significantly reduce the relative risk of DFS events in women aged <40 years (n=413) (HR 0.94, 95% CI 0.57-1.56), but did in women aged >40 years (n=1390) (HR 0.58, 95% CI 0.40-0.84) (Gnant, Mlineritsch *et al.* 2011), possibly due to inadequate suppression of ovarian function with goseralin in a younger cohort of patients.

The beneficial effect of zoledronic acid in a low oestradiol environment is also supported by the ZO-FAST trial (n=1065) that recruited postmenopausal women and randomized to early zoledronic acid+letrozole started at diagnosis, or delayed zoledronic acid started at the time of significant bone loss (median 12.8 months). Early zoledronic acid was associated with a 34% reduction in the risk of disease recurrence (local and distant) or death at 60 months, and the reduction in distant disease recurrence was primarily in bone, but also showed a trend to fewer recurrences outside bone (Coleman, de Boer *et al.* 2013). Moreover, the beneficial effect of early zoledronic acid versus delayed on overall survival in this postmenopausal population was driven by women who were >5 years since menopause or aged >60 years compared to recently postmenopausal women (HR 0.5 p=0.0224), supporting the theory that women with stable very low levels of oestradiol may be deriving the most benefit from adjuvant bisphosphonates both in bone and outside of bone.

Although baseline levels of individual hormones were not predictive of a response to zoledronic acid, they did appear to have a prognostic role in identifying patients at increased risk of bone and distant recurrence. Premenopausal pretreatment levels of FSH (<26IU/l) were borderline prognostic for a shorter time to bone recurrence. This finding is supported by clinical studies evaluating the presence of DTCs from breast cancer in the bone marrow at diagnosis. A large meta-analysis (n=4700) of the prevalance of bone marrow DTCs demonstrated that premenopausal patients had a significantly higher prevalence compared to postmenopausal women (premenopausal 32.7%, post menopausal 29.5% p=0.02)(Braun, Vogl *et al.* 2005), suggesting premenopausal bone may be more attractive to tumour cells. In addition, data on recurrence patterns of 6792 breast cancer patients entered into trials conducted by the International Breast Cancer Study Group showed that younger patients (<35 years) had significantly higher incidences of bone metastases occurring during the course of their disease, however when patients were categorized according to pre- or postmenopausal status there was no significant difference in rates of bone metastases (Colleoni, O'Neill *et al.* 2000). These data suggest that very young women, who would be expected to have low FSH levels, have a higher incidence of bone metastases. However, categorizing patients as premenopausal would include both young and older menstruating women and it is likely that average FSH levels in this group would be higher than women <35 years, with subsequent loss of a significant effect on bone recurrence rates. Smaller studies have both supported and refuted these results. Eskelinen *et al* correlated baseline FSH levels to DFS and OS in premenopausal (n=182) and postmenopausal (n=570) women treated for primary invasive breast cancer. There was a trend to a lower probability of survival with lower FSH levels in premenopausal women (Eskelinen, Norden *et al.* 2004). Pujol *et al* reported a prospective evaluation of baseline FSH in premenopausal (n=360) breast cancer patients and showed a trend for worse outcomes with higher FSH levels (Pujol, Daures *et al.* 2001). It is therefore not clear from existing evidence whether FSH is a true prognostic marker for DFS in breast cancer, however our data suggest that a low FSH may be more important as a prognostic marker specifically for bone recurrence. This requires further investigation, especially considering the data supporting the anti-tumour efficacy of adjuvant zoledronic in conjunction with goseralin in premenopausal (low FSH) patients (Gnant, Mlineritsch *et al*. 2011).

Postmenopausal levels of oestradiol (<50pmol/l) were borderline prognostic for a shorter time to distant (bone + non bone) recurrence which may be driven by increased non bone recurrence, suggesting that postmenopausal bone may be less attractive for DTCs and they may preferentially spread to non-skeletal sites. These data are supported by a large (n=9766) retrospective analysis of recurrence rates in postmenopausal ER+ve breast cancer patients treated with adjuvant endocrine therapy. An increased risk of distant but not locoregional recurrence with increasing age at diagnosis was found (van de Water, Seynaeve *et al*. 2013). Although baseline oestradiol was not measured in this study, serum levels would be expected to progressively fall with increasing age and may have contributed to the increase in distant recurrence. This study also showed less use of systemic chemotherapy with increasing age (van de Water, Seynaeve *et al*. 2013). The percentage of AZURE patients receiving chemotherapy+/- endocrine therapy was similar according to baseline oestradiol levels (Table 3.6), suggesting that time to distant recurrence was not influenced by differential administration of systemic therapy. These data imply that reproductive hormones may influence the survival of DTCs in the bone microenvironment. These DTCs, under hormonal influences, could subsequently form established bone metastases, or move to other distant sites. The effects of reproductive hormones on the bone microenvironment may be direct via modification of the size of the osteoblastic niche (Shiozawa, Havens *et al*. 2008), or indirect via interactions with paracrine tumour suppressors such as activin and TGFβ (Wilson, Holen *et al*. 2012). This requires further investigation in order to identify the cellular and molecular mechanisms.

In conclusion, biochemical menopausal categorisation should not replace, but may complement, clinical categorisation in a mixed heterogenous breast cancer population considered for adjuvant zoledronic acid. Selecting biochemically postmenopausal patients from women with a clinically unknown menopausal status or within 5 years of menopause, for adjuvant treatment with zoledronic acid may be possible, but is likely to require serial measurements rather than a single evaluation. The anti-tumour efficacy of zoledronic acid is more likely to be affected by ovarian hormones rather than pituitary

Table 3.5 Baseline characteristics according to oestradiol levels in the AZURE serum population

	Oestradiol <50pmol/l N(%)	Oestradiol ≥ 50pmol/l N(%)
Tumour stage		
T1	109 (33.9)	141 (29.3)
T2	163 (50.6)	237 (49.2)
T3	42 (13)	83 (17.2)
T4	8 (2.5)	21 (4.4)
Systemic therapy		
Endocrine therapy	10 (3.1)	2 (0.4)
Chemotherapy	77 (23.9)	104 (21.6)
Endocrine+chemotherapy	235 (73)	376 (78)
ER status		
Positive	243 (75.5)	376 (78)
Negative	78 (24.2)	104 (21.6)
Lymph node involvement		
0	3 (0.9)	13 (2.7)
1-3 nodes involved	202 (62.7)	282 (58.5)
≥ 4 nodes involved	117 (36.3)	185 (38.4)

hormones, but in our analysis no single level of individual hormone was predictive of a response to zoledronic acid. The concept of pretreatment levels of FSH and oestradiol having prognostic value in identifying patients at increased risk of bone or distant recurrence requires further investigation before this could be used to tailor adjuvant treatment.

Chapter 4. Neo-adjuvant zoledronic acid alters serum levels of activin, follistatin and TGFβ when added to standard chemotherapy for breast cancer.

An ANZAC sub-study.

4.1 Summary

As previously described in chapter 1, neo-adjuvant breast cancer clinical trials have suggested that zoledronic acid may have an anti-tumour effect, and can improve pathological response in breast tumours, when combined with chemotherapy. The efficacy of zoledronic acid in early breast cancer appears to be enhanced in certain subgroups including postmenopausal women and women with oestrogen receptor (ER) negative breast cancer.

Inhibin A is an ovarian hormone that declines to undetectable levels at the time of the final menstrual period. Inhibin A is a member of the TGFβ superfamily of signaling peptides, which includes activin and TGFβ. Activin and TGFβ are secreted by breast cancer cells, with the former peptide being a tumour suppressor, and the latter having both tumour suppressive and promoter actions depending on the stage of tumourigenesis. Activin is bound to follistatin, which neutralizes its biological activity, therefore to evaluate levels of 'biologically active' activin, follistatin levels must be concurrently assessed.

The ANZAC study randomised 40 women to receive zoledronic acid 4mg IV, 24 hours after the first cycle of FEC_{100} neo-adjuvant chemotherapy versus chemotherapy alone, to evaluate change in tumour apoptosis from baseline to day 5 in serial breast biopsies. Serum was collected at baseline, day 5 and 21 post administration of zoledronic acid. The effects of zoledronic acid on serum levels of activin, follistatin and TGFβ were evaluated in the overall population and in subgroups according to menopausal status and ER status of primary tumour.

Serum follistatin significantly fell in the overall study population from baseline to day 21 with the addition of zoledronic acid to chemotherapy. This change was driven by postmenopausal women, since premenopausal women showed no significant change in follistatin at day 21 with addition of zoledronic acid. Patients with ER-ve tumours had a significant fall in serum follistatin from baseline to day 5 with addition of zoledronic acid to chemotherapy, an effect not seen in patients with ER+ve tumours.

These novel data suggest that zoledronic acid can reduce serum follistatin, which antagonizes the anti-proliferative effects of activin, and this may lead to an enhanced anti-tumour effect in postmenopausal and ER-ve patients.

4.2 Introduction

There is a wealth of preclinical data demonstrating that zoledronic acid has direct anti-tumour effects both *in vitro* and *in vivo*, as discussed in chapter 1. Zoledronic acid has demonstrated a synergistic anti-tumour effect when used in combination with anthracycline chemotherapy. Maximal tumour cell apoptosis was achieved in ER+ve (MCF7) and ER-ve (MDA-MB-436) cell lines when zoledronic acid was given immediately after treatment with doxorubicin. (Neville-Webbe, Rostami-Hodjegan *et al*. 2005). This sequence dependent effect was confirmed *in vivo* in a sub-cutaneous xenograft model of ER-ve MDA-MB-436 tumours. Treatment with doxorubicin and clinically relevant doses of zoledronic acid (100μg/kg) simultaneously decreased tumour volumes compared to doxorubicin alone or zoledronic acid alone, However, doxorubicin followed 24 hours later by zoledronic acid resulted in almost total tumour regression (Ottewell, Monkkonen *et al*. 2008).

Zoledronic acid, when administered clinically at 4mg IV, results in a peak plasma concentration of ~2μM, which subsequently falls to ~0.1μM at 4 hours and <0.05μM at 8 hours (Spencer, Roberts *et al*. 2008). In situ primary breast tumours, will therefore be exposed to a low concentration of zoledronic acid at the time of administration. Despite this there is emerging clinical evidence that, when combined with anti-cancer therapy, these low doses of zoledronic acid can exert an anti-tumour effect outside of bone.

Neo-AZURE was a retrospective evaluation of a subgroup of 205 women in the AZURE trial that received neo-adjuvant chemotherapy (CT) +/- zoledronic acid (zol). The primary endpoint was residual invasive tumour size (RITS). Zoledronic acid significantly reduced RITS when added to chemotherapy (RITS; ZOL+CT 15.5mm, CT 27.4mm p=0.006). Multivariate analysis demonstrated a trend for a higher efficacy of zoledronic acid in reducing RITS in ER-ve tumours vs ER+ve tumours (p=0.0609) (Coleman, Winter *et al*. 2010). These data have been supported by Aft *et al*, who randomized 119 patients to perioperative CT +/- ZOL q3/52 for 1 year. Disease free survival (DFS) and overall survival (OS) were not different between groups in the overall population, however, patients with ER-ve tumours had a significant reduction in disease recurrence and death with addition of zoledronic acid (DFS; HR=0.361, 95% CI 0.148 to 0.88: OS; HR= 0.375, 95% CI 0.143 to 0.985)(Aft, Naughton *et al*. 2012).

The reasons for the differential effect of zoledronic acid according to menopausal status and ER status demonstrated in (neo)adjuvant clinical trials have not been defined. The differences between a premenopausal woman and a postmenopausal woman have been extensively discussed previously (chapter 3), and for the remainder of my research I chose to focus on the role of inhibin A as a potential endocrine modifier of the anti-tumour efficacy of zoledronic acid. Inhibins are members of the TGFβ super-family of signaling peptides, are secreted by functioning ovaries, and are detectable in serum of premenopausal women but decline to undetectable levels in postmenopausal women as previously discussed in chapter 3. Inhibins are heterodimeric proteins consisting of a dimer of 2 subunits; an α subunit and either a βA or βB subunit to form the respective inhibin A or B. There is no known downstream intracellular signaling pathway for the inhibins (Bilandzic and Stenvers 2011), and thus the molecular mechanism of action is via inhibition of receptor binding of other TGFβ superfamily ligands including activin and TGFβ, which are abundant in many tissues, including bone (Ogawa, Schmidt *et al.* 1992; Iqbal, Sun *et al.* 2009).

Both activin and TGFβ1 have paracrine tumour modifying effects in breast cancer. Activin is secreted by breast cancer cell lines *in vitro* and inhibits proliferation (Kalkhoven, Roelen *et al.* 1995; Cocolakis, Lemay *et al.* 2001). In addition, in clinical breast tumour samples, as grade of tumour increases, there is loss of functioning activin pathways (Jeruss, Sturgis *et al.* 2003). Activin is bound to follistatin, a single chain glycosylated polypeptide, which neutralizes the biological activity of activin thus preventing its tumour suppressive activity (Shimonaka, Inouye *et al.* 1991). TGFβ1 is secreted by breast cancer cell lines *in vitro*, and has been shown to have both an inhibitory and promoting effect on breast tumour development in mouse models (Yang, Dukhanina *et al.* 2002; Forrester, Chytil *et al.* 2005).

Inhibin therefore has relevant effects in breast cancer via its modification of these associated paracrine factors, However, it is not known if zoledronic acid can directly affect activin, follistatin or TGFβ1 in breast cancer, or if the effects of zoledronic acid on these factors may be different in an inhibin rich (premenopausal) vs inhibin poor (postmenopausal) endocrine environment.

ANZAC (A Randomised phase II feasibility study investigating the biological effects of the **A**dditio**N** of **Z**oledronic **A**cid to neo-adjuvant combination **C**hemotherapy on invasive breast cancer) was a prospective randomized phase II feasibility study designed and run by Dr Matthew Winter at Sheffield University 2009-2011. ANZAC was designed to evaluate the synergistic anti-tumour effects of chemotherapy and zoledronic acid in 40 breast cancer patients (pre- and postmenopausal) receiving zoledronic acid 4mg IV 24 hours after receiving first cycle of neo-adjuvant FEC_{100} chemotherapy. The primary objectives of this study were to evaluate change in apoptosis between the diagnostic core biopsy and an additional biopsy at day 5-post treatment. Secondary objectives included the evaluation of ki67 on repeat core biopsies and serum angiogenesis biomarkers at baseline, day 5 and day 21. Stored serum allowed evaluation of serum activin A, TGFβ1 and follistatin at baseline, and subsequent time points after administration of CT+/- zoledronic acid, in premenopausal/high inhibin (n=18) and postmenopausal/low inhibin (n=22) patients. Further evaluation of serum levels, according to ER status of the primary breast tumour, was also possible since this information was collected at baseline as part of the main study.

To my knowledge, there have been no clinical studies evaluating changes in serum levels of activin, TGFβ1 or follistatin with addition of zoledronic acid to neo-adjuvant chemotherapy in early breast cancer. The new information in this chapter shows that zoledronic acid can affect serum levels of follistatin, activin and TGFβ1 when added to standard neo-adjuvant chemotherapy. The fall in follistatin at day 21 was driven by postmenopausal women, who also have a concurrent increase in serum activin, thus increasing the bioavailability of this tumour suppressor in serum in this subgroup. There was also an early fall in follistatin at day 5 in patients with ER-ve tumours, However, the numbers in these analyses are small and require further investigation in a larger cohort of patients.

4.3 Aims

The overall purpose of this chapter was to assess serum samples from the ANZAC study to evaluate whether zoledronic acid, whan added to standard chemotherapy, can alter serum levels of activin, follistatin and TGFβ1, and if menopausal status or oestrogen receptor (ER) status can modify this. The following aims were set;

1. Evaluate if zoledronic acid alters levels of activin, follistatin or TGFβ1 in the overall study population.

2. Evaluate if zoledronic acid alters serum levels of activin, follistatin or TGFβ1 differentially, according to menopausal status of patients.

3. Assess if zoledronic acid alters serum levels of activin, follistatin or TGFβ1 differentially, according to ER status of primary tumours

4. Determine if serum levels of activin, follistatin or TGFβ1 correlate with growth index (ki67/apoptosis) of primary breast tumours.

4.4 Patients and Methods

4.4.1 Ethical approval

This study was approved by the South Yorkshire National Research Ethics committee, with a favorable outcome obtained on the 4th August 2011 (REC ref no; 11/YH/0259).

4.4.2 Study population

This study was a retrospective analysis of serum from patients recruited into the ANZAC trial. ANZAC was a single centre open label randomized phase II feasibility study. Patients had a histological diagnosis of invasive breast cancer and were recommended for neo-adjuvant chemotherapy. Standard neo-adjuvant chemotherapy was FEC_{100} (Fluorouracil 500mg/m^2, Epirubicin 100mg/m^2 and Cyclophosphamide 500mg/m^2 all administered day 1 of a 3 week cycle) x3 followed by Docetaxel (100mg/m^2 day 1 of a 3 week cycle) x3. Patients were randomized on a 1:1 basis to addition of zoledronic acid 4mg IV to be administered 24 hours after the first cycle of FEC_{100} (Fig 4.1). Repeat core biopsies were scheduled for day 5 and 21 and serum was collected at baseline, and at the time of repeat core biopsies. For this retrospective analysis, serum was evaluated for the proteins of interest, and any correlations to tumour proliferation characteristics were done using previously scored immunohistochemistry results from tumour sections from the original ANZAC study, sections were not re-scored for the purpose of this sub-study

4.4.3 Inclusion and exclusion criteria

Inclusion and exclusion criteria for the main ANZAC trial have been previously published, but are summarized in table 4.1 (Winter, Wilson *et al.* 2013).

Inclusion criteria for retrospective study;

- Recruited into the ANZAC trial (A randomized phase II feasibility study investigating the biological effects of the **A**dditio**N** of **Z**oledronic **A**cid to neo-adjuvant combination **C**hemotherapy on invasive breast cancer). EudraCT number; 2007-001526-27.

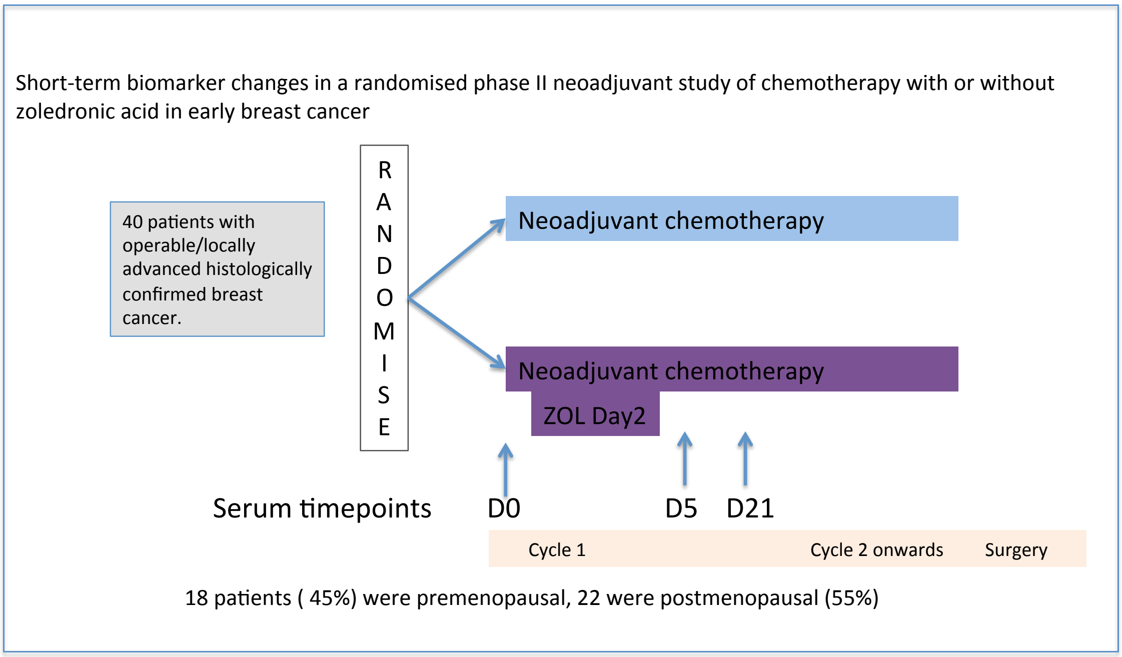


Figure 4.1 ANZAC trial schematic.

40 patients with histologically confirmed breast cancer and recommended for neo-adjuvant therapy were randomised to receive first cycle of FEC_{100} chemotherapy +/- addition of zoledronic acid 24 hours post chemotherapy. Serum was collected at baseline, day 5 and day 21 and was available from the majority of patients for this sub-study.

Table 4.1 Inclusion and exclusion criteria for the ANZAC study

Inclusion criteria	Exclusion criteria
Histological diagnosis of invasive breast cancer	T0 T1 tumours
T2 tumour or above	Previous chemotherapy or radiotherapy to treated breast
Recommended for neo-adjuvant chemotherapy	Previous diagnosis of malignancy unless contralateral breast cancer off all treatment for >6 months, disease free for 5 years, non melanomatous skin cancer or carcinoma of the uterine cervix treated curatively
Any hormone or HER2 status	Serum creatinine >1.5 x upper limit of normal reference range or calculated creatinine clearance <40mls/min
Age >18	Prior treatment with bisphosphonates in the last year
Reliable method of contraception if appropriate	Concurrent tamoxifen or aromatase inhibitors
WHO PS 0-2	On anticoagulation including warfarin or coumarin derivatives
Confirmed core biopsy at baseline and consent to additional core biopsy	Known hypersensitivity to bisphosphonates
Written informed consent	Current active dental problems
	Recent or planned dental surgery
	Pregnant or lactating women
	Cardiac dysfunction that precludes anthracycline chemotherapy
	Males
	Unwilling to have repeat breast biopsies

- Serum stored at Cancer Clinical Trials Unit, Sheffield, UK and collected according to the ANZAC study laboratory protocol.

- Confirmed written consent to storage and analysis of serum.

Exclusion criteria for retrospective study;

- No written consent to storage and analysis of serum

- Inadequate volume of serum for analysis

4.4.4 Storage and transport of ANZAC serum

All serum samples were stored at the Cancer Clinical Trials Unit, Weston Park Hospital, Sheffield, UK. Samples were stored at -80°C. All samples sent for analysis had undergone ≤1 freeze thaw cycle. Transport of samples between the trials unit and the medical school laboratory was on dry ice to prevent numerous freeze thaw cycles prior to analysis.

4.4.5 Analyses of proteins.

All protein analyses were performed by myself, with supervision from Mrs Alyson Evans, a senior technician in the laboratory team. Inhibin A, activin A, follistatin and TGFβ1 were analysed on manual ELISA plates purchased from R&D (see section 2.2.6 for minimum detection limits). Serum was fully defrosted prior to use and aliquoted into quantities specific for each ELISA to reduce freeze thaw cycles. All samples from individual patients were analysed in the same run. Standard curves were included on each plate; samples were analysed in duplicate with CVs <15%.

FSH analysis was performed on the automated Roche 602 Elecsys electrochemiluminescence immunoassay at the Clinical Chemistry Laboratory Sheffield Teaching Hospitals. FSH levels were standardized to the second International Reference Preparation 78/549. For assay precision and quality control data see chapter 3, table 3.1.

4.4.6 Data collection from ANZAC database, and correlation with serum measurements.

Demographic, treatment and outcome data for all patients recruited to ANZAC was held on a password protected database by the Chief Investigator, Dr Matthew Winter. Permission for access to this database was given. The following data was extracted for the analyses;

1. Patient characteristics at randomisation;

- Trial number
- Age
- Clinical menopausal status; premenopausal vs. postmenopausal

2. Disease characteristics at randomization;

- ER status; Positive vs. negative

3. Treatment characteristics;

- Randomised to: Interventional arm (zoledronic acid) or control arm (no zoledronic acid)

4. Disease outcomes;

- Growth index of tumours at baseline, day 5 and day 21. Growth index was a ratio of Ki67/apoptosis (%) measured on the biopsy samples as part of the ANZAC trial, methodology described in the published paper (Winter, Wilson *et al*. 2013)

The data collected on the serum analysis was entered in an Excel spreadsheet and combined with the data extracted from the main ANZAC database.

4.4.7 Statistical methodology

All statistical analysis was performed by myself using Graphpad PRISM v6.0 software. For comparison of treatment groups, the nonparametric Mann Whitney U test was performed. For correlation analysis, Spearmen's nonparametric correlation coefficient was performed.

4.4.8 Funding and publication

The cost of the serum analyses were funded by a grant from Weston Park Cancer Charity awarded to myself, as main applicant, on the 27th March 2012. The results of this analysis have been published in Clinical Cancer Research in 2013 (Winter, Wilson *et al*. 2013).

4.5 Results

4.5.1 Patient samples available for analysis.

Baseline demographic data according to treatment received has previously been published (table 4.2). For these analyses, >95% of patients had sufficient stored serum for evaluation of activin, follistatin and TGFβ at baseline, day 5 and day 21. All serum levels were within assay detection ranges.

Baseline levels of activin, follistatin, TGFβ and inhibin A were not significantly different according to treatment group or ER status. Baseline activin, follistatin and TGFβ were not significantly different according to menopausal status, However, as expected, inhibin A was significantly different. Baseline median (range) inhibin A levels in pre- and postmenopausal patients were 27.9pg/ml (5.1-171.3) and 5.8pg/ml (0.7-17.6) respectively, with a persistent lower level in postmenopausal women at all other timepoints (Pre- vs. postmenopausal, Day 5; 21.90 pg/ml (4.78-70.5) vs. 7.6 pg/ml (0.9-29.8), Day 21; 11.95 pg/ml (1.4-61.4) vs. 5.4 pg/ml (0.1-14.9). This was statistically significant with mean difference in AUC (pre-post): 16.9 pg/ml, 95% CI: 9.1, 24.6, P<0.001.

Median inhibin A levels significantly declined in premenopausal patients from baseline to day 21 (p=0.0016), suggesting that chemotherapy may decrease serum inhibin A levels in premenopausal patients (Fig 4.2)

4.5.2 Effect of zoledronic acid in the overall study population.

4.5.2.1 Serum activin

Figure 4.3 shows the change in serum activin levels over time for each individual patient treated with CT alone or CT+ZOL. There was a consistent fall in serum activin from baseline to day 5 in 98% of all patients. By day 21, 45% (9/20) and 65% (13/20) of patients treated with CT alone or CT+ZOL had recovered activin levels to higher than baseline (Fig 4.4). Addition of zoledronic acid to chemotherapy did not significantly affect the changes in activin from baseline to day 5 or baseline to day 21 (Median change from baseline pg/ml; Day 5, CT -116 (IQR -166 to -85) CT+ZOL -98 (IQR -142 to -69) p value 0.2853. Day 21, CT -2.5 (IQR -50 to +38) CT+ZOL +44.6 (IQR -9 to +87) p value 0.0764) suggesting chemotherapy may be driving the changes in serum activin (Table 4.4).

Table 4.2. Baseline clinico-pathological characteristics of patients enrolled in the ANZAC study.

	Chemotherapy alone n=20 (%)	Chemotherapy + Zoledronic acid n=20 (%)
Median age years (IQR)	49 (46 – 57)	51 (46 – 56)
Tumour stage		
T2-T3	17 (85.0)	16 (80.0)
T4	1 (5.0)	1 (5.0)
T4d	2 (10.0)	3 (15.0)
Menopausal status		
Pre	11 (55.0)	11 (55.0)
Post	9 (45.0)	9 (45.0)
ER status		
Positive	14 (70.0)	16 (80.0)
Negative	6 (30.0)	4 (20.0)
HER2 status		
Positive	8 (60.0)	9 (45.0)
Negative	12 (40.0)	11 (55.0)

Table reproduced with permission from Dr Matthew Winter. MD thesis August 2011; Translational and clinical evaluation of chemotherapy and zoledronic acid in early invasive breast cancer, Sheffield University

ER status was defined as positive based on a McCarthy’s H score ≥50

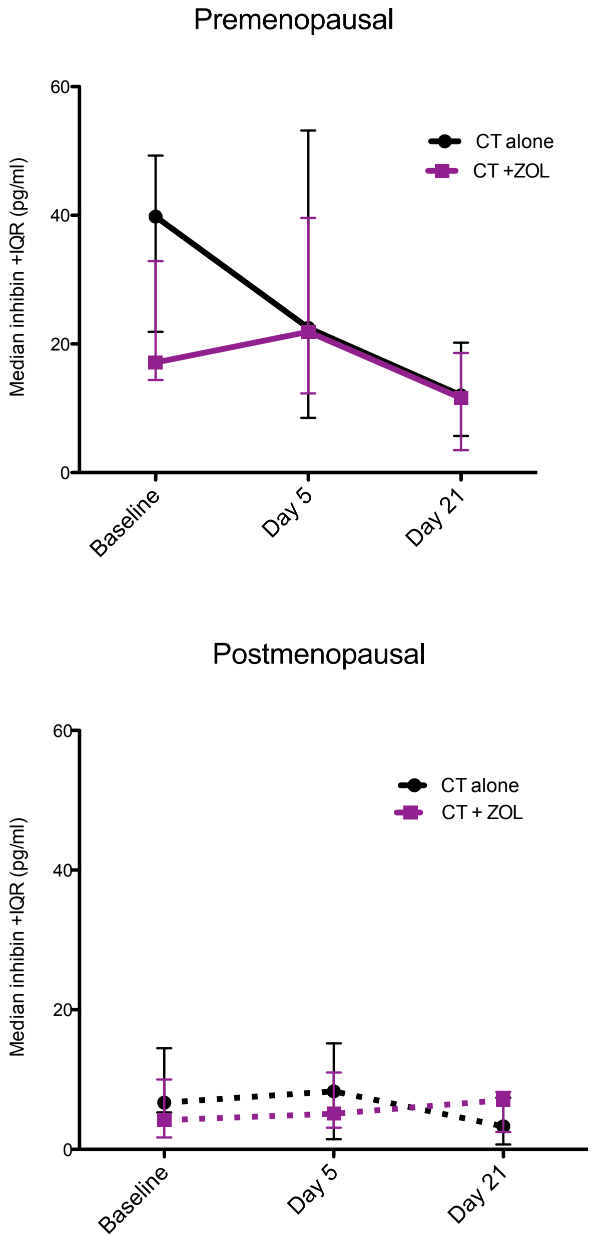


Figure 4.2 Effect of menopausal status on serum inhibin A levels.

Data represents median +IQR serum inhibin A levels at baseline, day 5 and day 21 in pre- and postmenopausal women treated with chemotherapy (CT) alone or CT+ Zoledronic acid (ZOL). Mean difference in area under the curve analysis for pre and postmenopausal women p value<0.001.

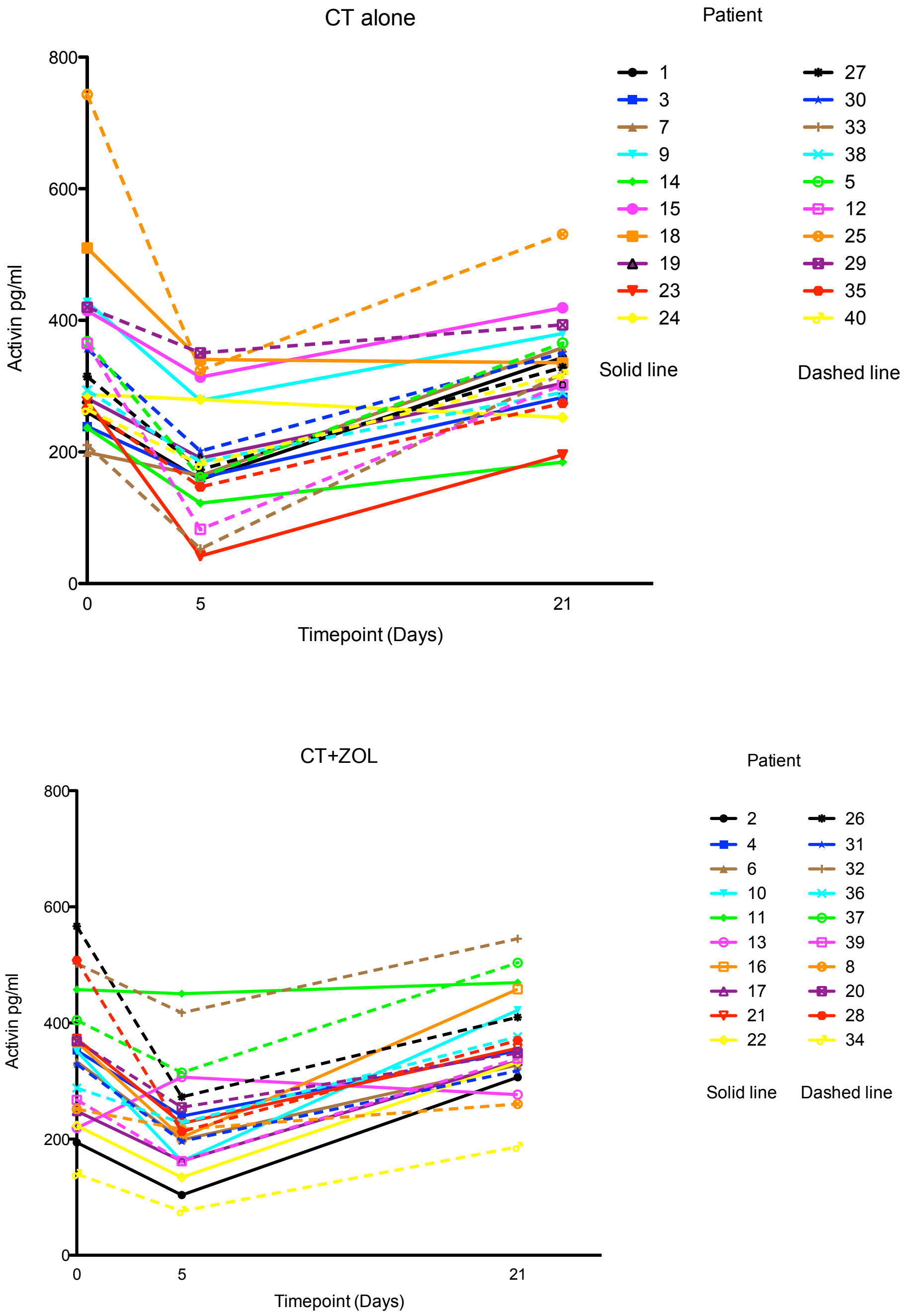


Figure 4.3 Changes in serum activin levels over time according to treatment received.

Data represents serum levels per patient to demonstrate change in serum level over time in patients receiving chemotherapy (CT) alone (top) and chemotherapy plus zoledronic acid (CT+ZOL) (bottom).

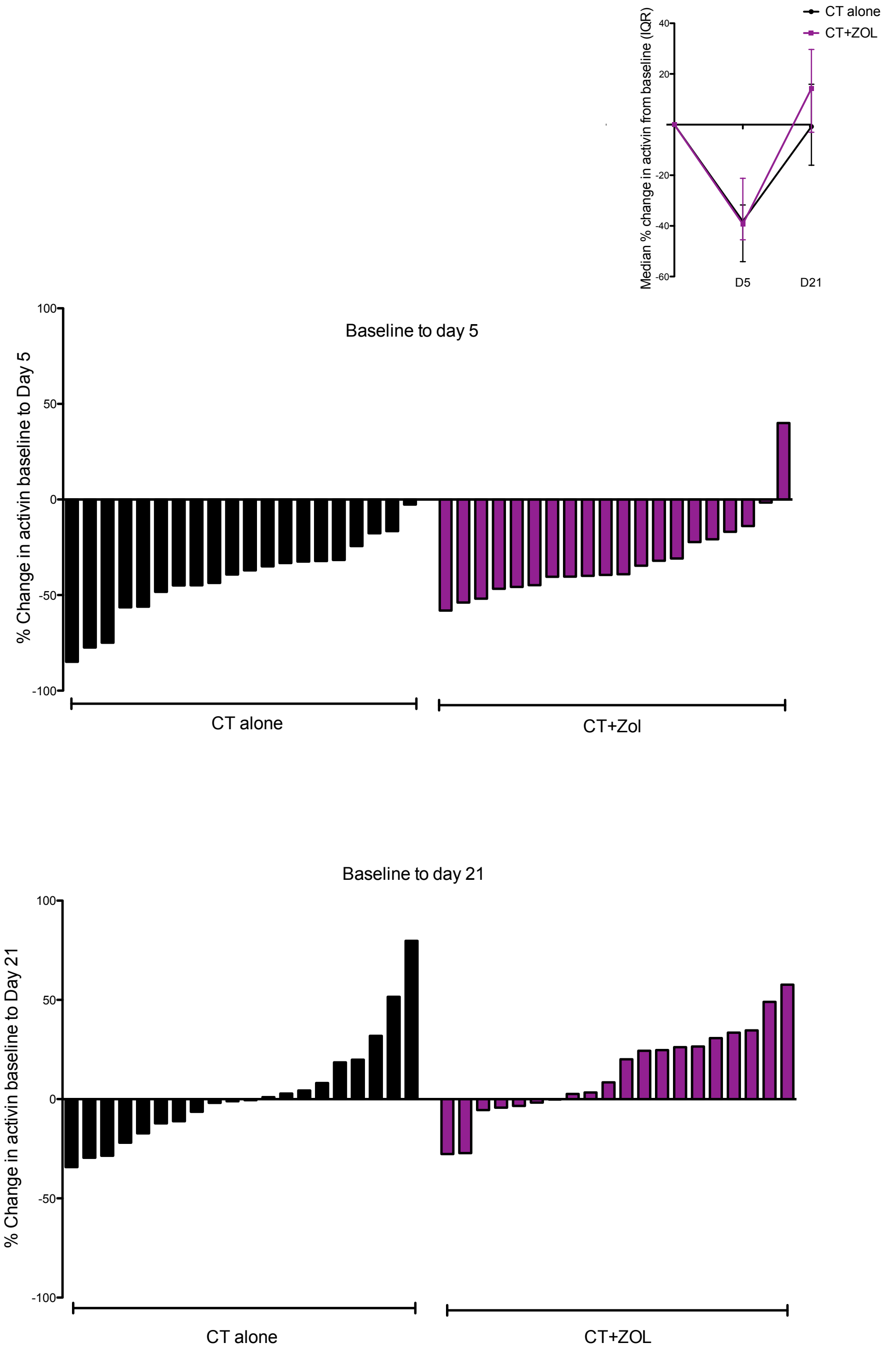


Figure 4.4. Percentage change in serum activin from baseline for individual patients.

Data represent individual percentage change for individual patients from baseline to day 5 (top) and baseline to day 21 (bottom). Inset represents median percentage change +IQR per treatment group.

Table 4.4 Changes in serum activin from baseline to day 5 and baseline to day 21 according to treatment received in the overall study population.

Data represent median (IQR) change from baseline, Mann Whitney U test to compare CT with CT+ZOL for each time-point measurement.

Time-point measurement of Activin	Treatment group	Median change	IQR	P value
Baseline to day 5 pg/ml	**CT CT+ZOL**	**-116 -98**	**-166 to -85 -142 to -69**	**0.2853**
Baseline to day 5 %	**CT CT+ZOL**	**-38.1 -39.2**	**-54 to -31 -45 to -21**	**0.3793**
Baseline to day 21 pg/ml	**CT CT+ZOL**	**-2.5 +44.6**	**-50 to +38 -9 to + 87**	**0.0764**
Baseline to day 21 %	**CT CT+ZOL**	**-0.07 14.26**	**-16 to 15.9 -3 to +29**	**0.081**

4.5.2.2 Serum follistatin

Figure 4.5 shows the change in serum follistatin over time for each individual patient treated with CT alone and CT +ZOL. The changes in serum follistatin from baseline to day 5 were similar in both treatment groups with 45% (9/20) and 60% (12/20) in the CT and CT+ZOL groups showing a decrease in follistatin, and the remainder of patients showing either no change or an increase in follistatin. At day 21 however, zoledronic acid appeared to be having an effect on serum follistatin, with more patients treated with CT+ZOL (80%) showing a decrease in follistatin compared to baseline than CT alone (40%)(Fig 4.6). Median change in follistatin from baseline to day 21 was significantly different between the two treatment groups (Median change pg/ml; CT alone 94.8 (IQR -173 to 432), CT+ZOL group -348 (IQR -886 to +3.5, p value 0.004) (Table 4.5). These data suggest that zoledronic acid, rather than chemotherapy, may be affecting serum follistatin at day 21.

4.5.2.3 Serum TGFβ1

Figure 4.7 shows the change in serum TGFβ1 over time for each individual patient treated with CT alone and CT+ZOL. Changes in TGFβ1 from baseline to day 5 showed a similar pattern to follistatin in that changes were similar between both treatment groups, with 60% (12/20) and 50% (10/20) in the CT and CT+ZOL groups having a decrease in TGFβ. At day 21 however, zoledronic acid appeared to be affecting serum TGFβ1 with more patients treated with CT+ZOL (85%) showing an increase in serum TGFβ1 compared to baseline than CT alone (60%) (Fig 4.8). Median change in TGFβ1 from baseline at day 21 was significantly different between the two treatment groups (Median change pg/ml; CT alone 5025 (IQR -891 to 9293), CT+ZOL 12259 (IQR +5346 to +17934) p value 0.0294)(Table 4.6). These data suggest that zoledronic acid may be affecting TGFβ1 levels at day 21.

All together, these data suggest that chemotherapy drives changes in serum activin however, zoledronic acid may be directly affecting serum levels of follistatin and TGFβ1 21 days post administration.

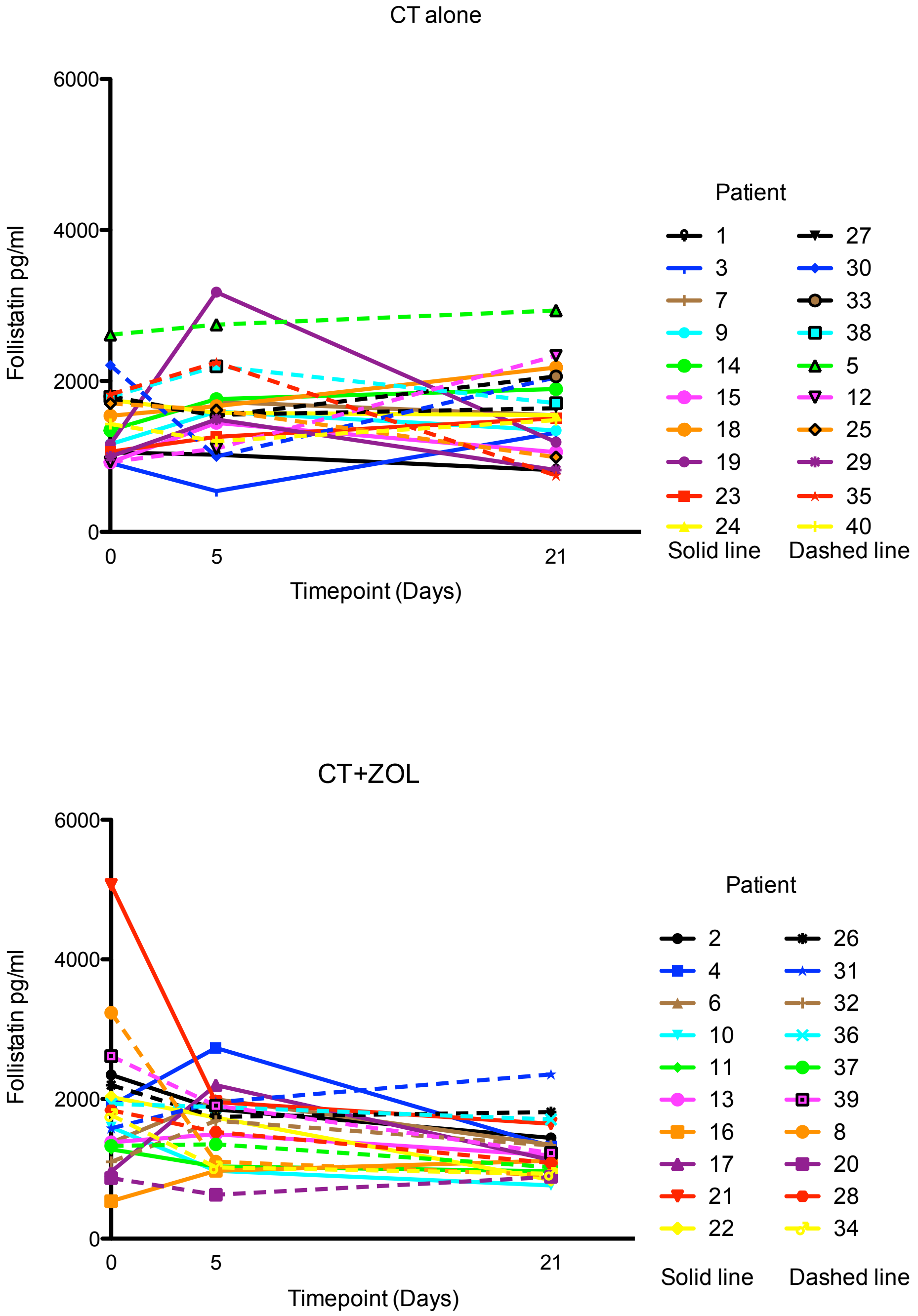


Figure 4.5 Changes in serum follistatin over time according to treatment received.

Data represents serum levels per patient to demonstrate change in serum level over time in patients receiving chemotherapy alone (top) and chemotherapy plus zoledronic acid (bottom).

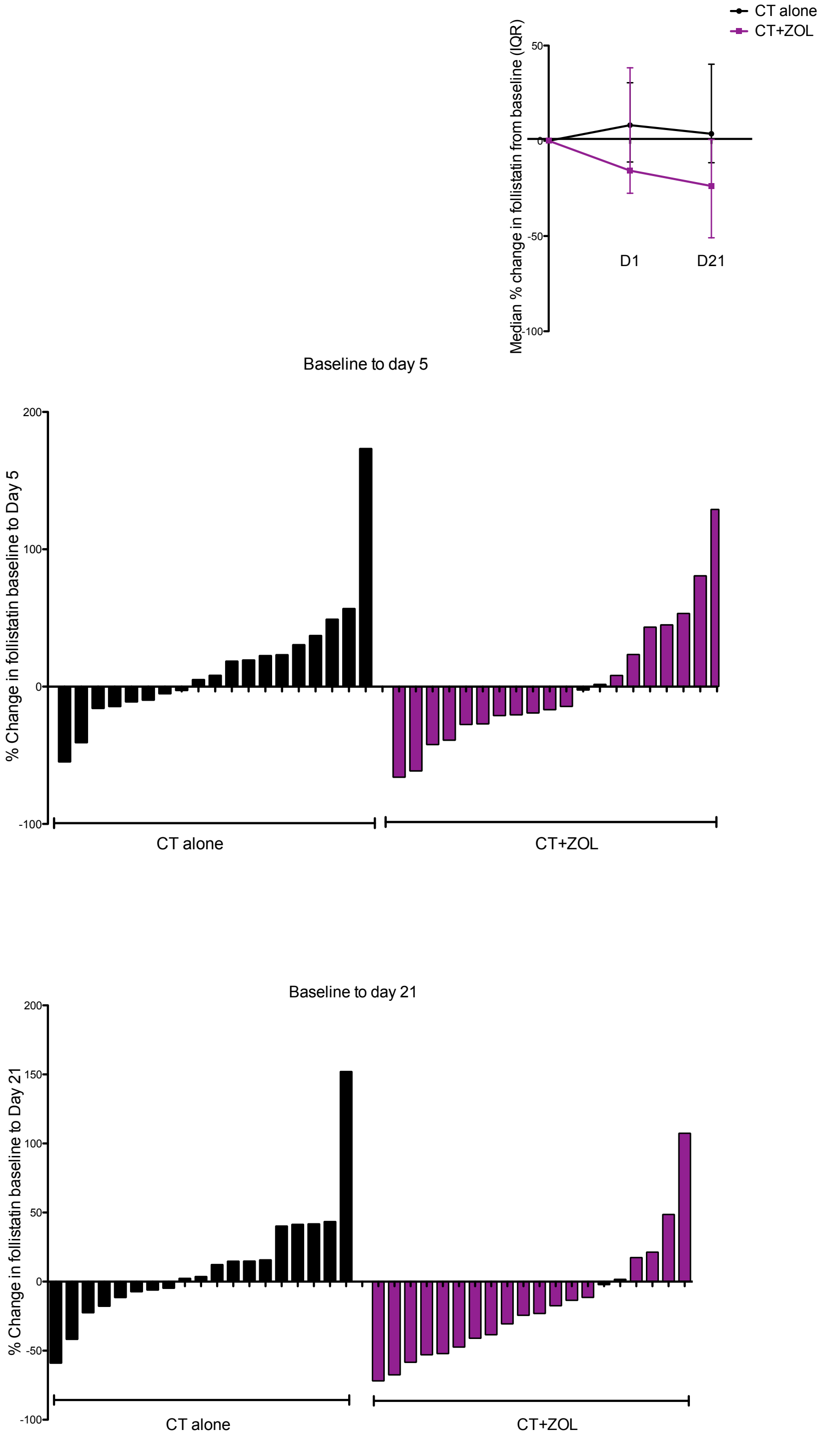


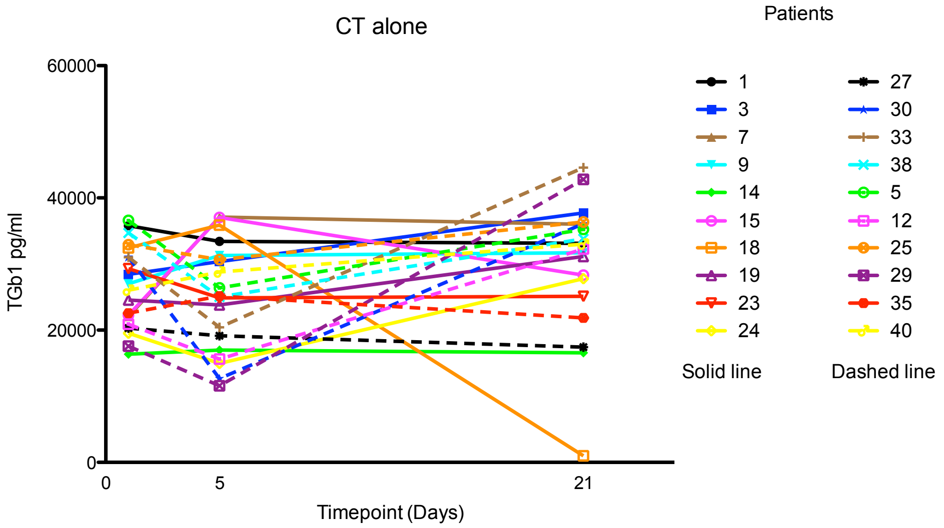
Figure 4.6. Percentage change in serum follistatin from baseline for individual patients.

Data represents individual percentage change for each patient from baseline to day 5 (top) and baseline to day 21 (bottom). Inset represents median percentage change +IQR per treatment group.

Table 4.5 Changes in serum follistatin from baseline to day 5 and baseline to day 21 according to treatment received for the overall study population.

Time-point measurement of Follistatin	Treatment group	Median change	IQR	P value
Baseline to day 5 pg/ml	**CT CT+ZOL**	**+133 -242**	**-193 to 423 -591 to 418**	**0.0962**
Baseline to day 5 % change	**CT CT+ZOL**	**+8.2 -15.61**	**-11 to 30 -27 to 38**	**0.1914**
Baseline to day 21 pg/ml	**CT CT+ZOL**	**94.8 -348.2**	**-173 to 432 -886 to 3.5**	**0.004***
Baseline to day 21 % change	**CT CT+ZOL**	**3.68 -23.71**	**-11 to 40.2 -50 to 0.73**	**0.0205***

Data represents median (IQR) change from baseline. Mann Whitney U test to compare CT with CT+ZOL for each time-point measurement. * p value <0.05



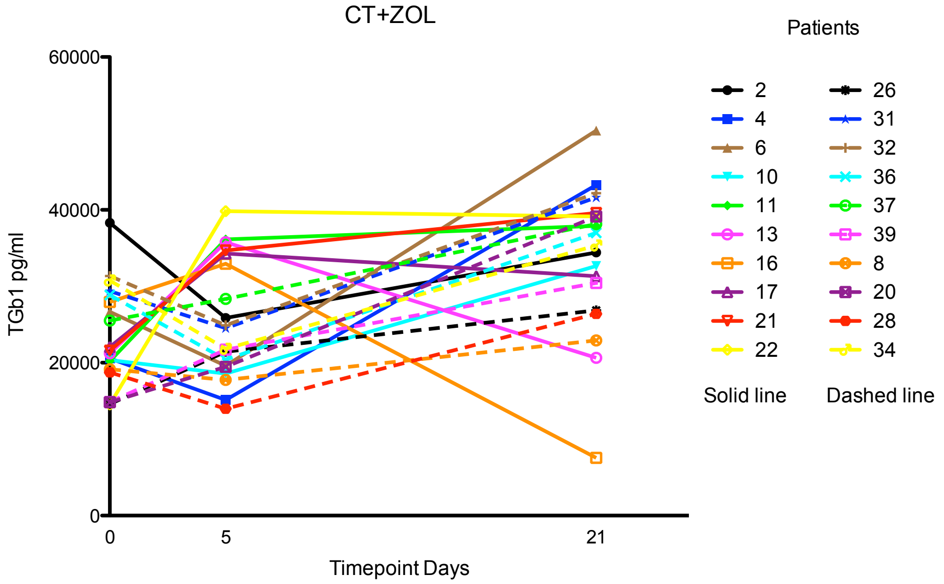


Figure 4.7 Changes in serum TGFβ1 time accoding to treatment received.

Data represents serum levels per patient to demonstrate change in serum level over time in patients receiving chemotherapy alone (top) and chemotherapy plus zoledronic acid (bottom).

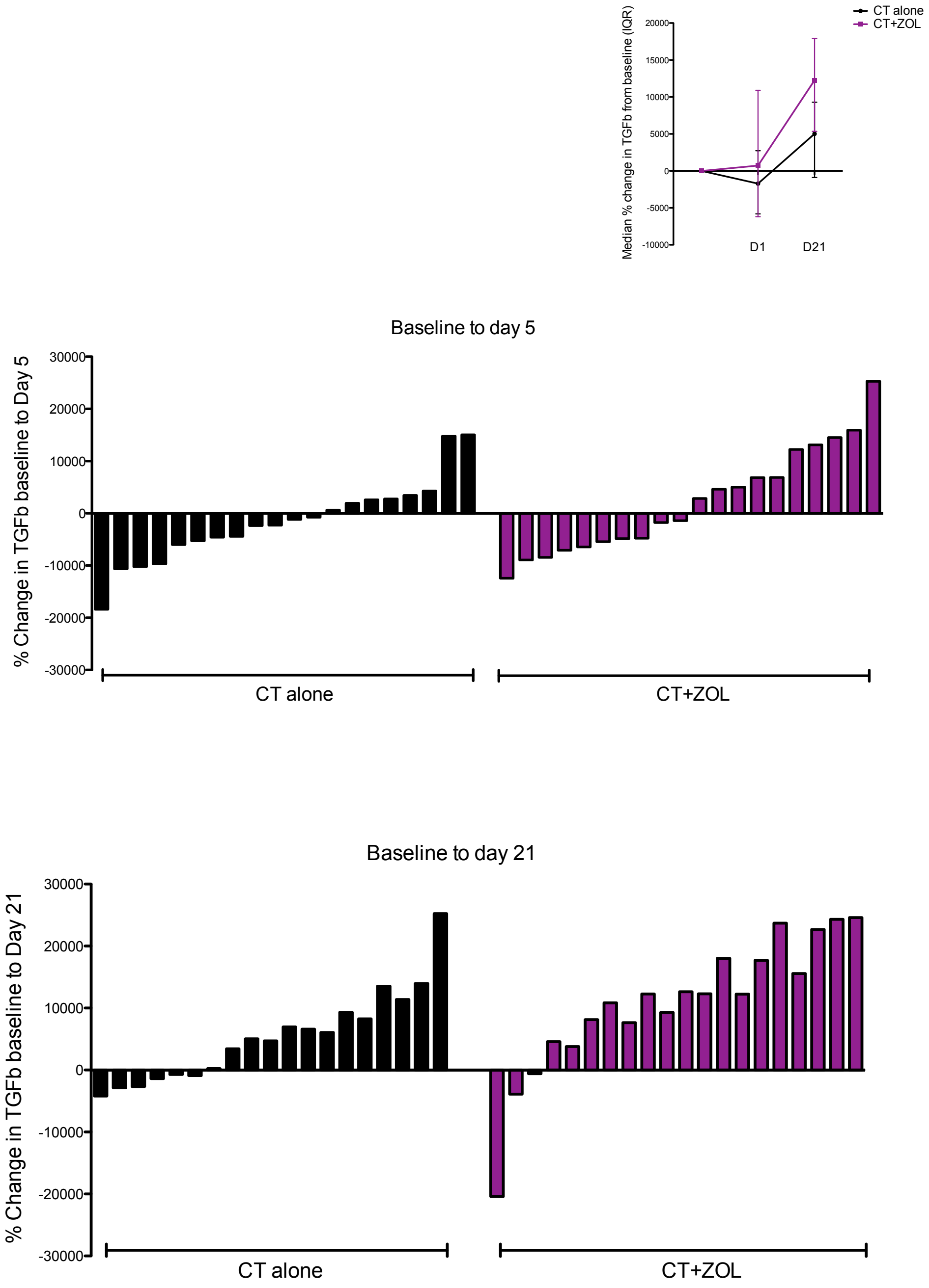


Figure 4.8. Percentage change in serum TGFβ1 from baseline for individual patients

Data represents individual percentage change for each patient from baseline to day 5 (top) and baseline to day 21 (bottom). Inset represents median percentage change +IQR per treatment group.

Table 4.6 Changes in serum TGFβ1 from baseline to day 5 and baseline to day 21 according to treatment received in the overall study population.

Time-point measurement of TGFb1	Treatment group	Median change	IQR	P value
Baseline to day 5 pg/ml	CT CT+ZOL	-1716 727	-5815 to 2729 -6187 to 10900	0.3369
Baseline to day 5 % change	CT CT+ZOL	-6.1 1.9	-27.2 to 10.6 -26.1 to 53.3	0.3104
Baseline to day 21 pg/ml	CT CT+ZOL	5025 12259	-891 to 9293 5346 to 17934	0.0294*
Baseline to day 21 % change	CT CT+ZOL	17.3 45.7	-3.1 to 42.3 21.8 to 88.56	0.0205*

Data represents median (IQR) change from baseline. Mann Whitney U test to compare CT with CT+ZOL for each time-point measurement. * p value<0.05.

4.5.3 Exploratory analysis of the effect of zoledronic acid on serum protein levels according to menopausal status and ER status.

4.5.3.1 Menopausal status

22 patients were classified as clinically premenopausal and 18 patients were postmenopausal. Clinical menopausal status was confirmed biochemically by measurement of baseline FSH. Median (range) FSH in clinically pre- and postmenopausal patients was 6.8 IU/l (1.8-30.7) and 68.4 IU/l (28-103.2) respectively (Fig 4.9). Only 1 premenopausal patient had an FSH above the assay specific cut point for postmenopausal (26.7 IU/l), However, baseline inhibin A in this patient was 15.5 pg/ml and therefore consistent with a premenopausal status.

Figure 4.10 shows the serum levels of activin, follistatin and TGFβ1 in pre- and postmenopausal patients according to treatment received. At day 5 there was no significant difference in the serum levels of any of the proteins of interest according to treatment received in pre- or postmenopausal patients. However, zoledronic acid appeared to differentially alter serum levels of both activin and follistatin from baseline to day 21 according to menopausal status of the patient.

Postmenopausal women showed an increase in serum activin at day 21 compared to baseline with addition of zoledronic acid to chemotherapy, an effect not seen in premenopausal women (median change from baseline to day 21 pg/ml; premenopausal women CT alone -1.8 (IQR -47.6 to +83.1), CT+ZOL group -0.6 (IQR -20.3 to 85.9) p=0.07, postmenopausal women CT alone -6.7 (-128.3 to 10.65), CT+ZOL 70.9 (IQR 27.1 to 100.2) p=0.004) (Table 4.7).

Postmenopausal women also showed a decrease in serum follistatin at day 21 compared to baseline with addition of zoledronic acid to chemotherapy, an effect not seen in premenopausal women (median change from baseline to day 21pg/ml; Premenopausal women CT alone -85 (IQR -238 to +182), CT+ZOL -384 (IQR -1186 to +167) p=0.18, postmenopausal women CT alone 321 (IQR -39 to +925), CT+ZOL -312 (IQR -793 to -86) p=0.004) (Table 4.7).

These data show that the addition of zoledronic acid to chemotherapy causes significant changes from baseline to 21 in activin (↑) and follistatin (↓) compared to chemotherapy alone, in postmenopausal women only. This suggests the significant fall in serum

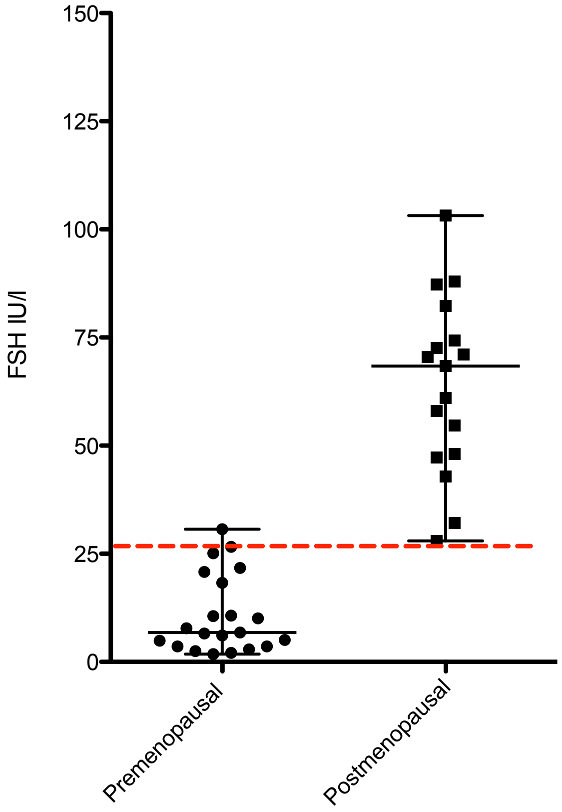


Figure 4.9 Serum follicle stimulating hormone according to menopausal status

Data represent serum values of FSH for all patients classifies as clinically pre- or postmenopausal. Red dotted line represents cut point for assay to define postmenopausal i.e. serum levels above red line defines patients biochemically as postmenopausal (FSH >26IU/l).

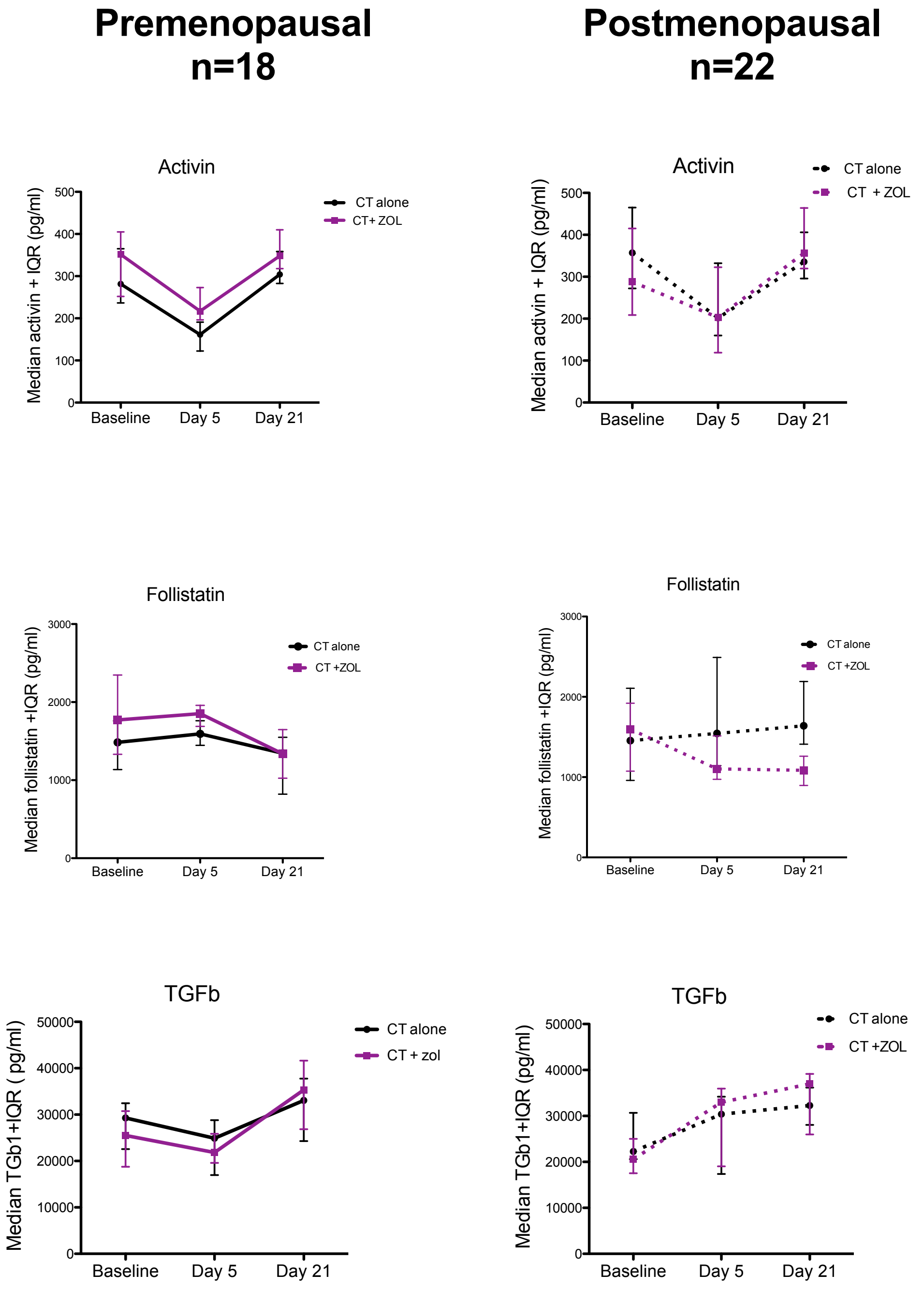


Figure 4.10 Changes in serum activin, follistatin and TGFβ1 according to menopausal status and treatment received.

Data represents the median +IQR at each time-point in premenopausal women (left) and postmenopausal women (right).

Table 4.7 Changes in serum activin, follistatin and TGFβ1 according to menopausal status.

Menopausal status + measured factor	Time-point	Treatment	Median Change pg/ml	IQR	P value
Premenopausal Activin	**Baseline to D5**	**CT CT+ZOL**	**-108.9 -113.7**	**-157.7 to -77.4 -189.4 to -85.9**	**0.84**
	Baseline to D21	**CT CT+ZOL**	**-1.8 -0.6**	**-47.6 to +83.1 -20.3 to 85.9**	**0.07**
Postmenopausal Activin	**Baseline to D5**	**CT CT+ZOL**	**-141.5 -89.1**	**-202.6 to -92.3 -125.6 to -61.9**	**1.0**
	Baseline to D21	**CT CT+ZOL**	**-6.7 70.9**	**-128.3 to 10.65 27.1 to 100.2**	**0.004***
Premenopausal Follistatin	**Baseline to D5**	**CT CT+ZOL**	**404.6 -291**	**-89 to +432 -709 to +586**	**0.23**
	Baseline to D21	**CT CT+ZOL**	**-85 -384**	**-238 to +182 -1186 to +167**	**0.18**
Postmenopausal Follistatin	**Baseline to D5**	**CT CT+ZOL**	**133.4 -239**	**-317 to +961 -466 to +273**	**0.43**
	Baseline to D21	**CT CT+ZOL**	**321 -312**	**-39 to +925 -793 to -86**	**0.004***
Premenopausal TGFβ1	**Baseline to D5**	**CT CT+ZOL**	**-2352 -4761**	**-10206 to +2594 -7070 to +6853**	**0.69**
	Baseline to D21	**CT CT+ZOL**	**+2627 +12256**	**-1697 to +8608 +4580 to +15567**	**0.11**
Postmenopausal TGFβ1	**Baseline to D5**	**CT CT+ZOL**	**-1155 +5006**	**-4918 to +9537 -3600 to +15200**	**0.34**
	Baseline to D21	**CT CT+ZOL**	**6066 12309**	**1269 to 10333 3786 to 23494**	**0.16**

Data represents median (IQR) change from baseline to day 5 (D5) and day 21 (D21). Mann Whitney test for significance between CT and CT+ZOL. *p value <0.05

follistatin seen in the overall population, with addition of zoledronic acid, is being driven by the postmenopausal patients

4.5.3.2 Oestrogen receptor (ER) status

30 patients had ER+ve primary breast tumours and 10 patients had ER –ve primary breast tumours at diagnosis.

Figure 4.11 shows the serum levels of activin, follistatin and TGFβ1 in ER+ve and ER-ve patients according to treatment received. At day 5 there was a significant difference in serum follistatin in ER-ve patients treated with CT or CT+ZOL, with follistatin being lower in the latter. This effect was not seen in patients with ER+ve tumours (median change baseline to day 5, pg/ml; ER-ve patients CT alone 156 (IQR -123 to -440), CT+ZOL -528 (IQR -1787 to -257) p=0.0095; ER+ve patients CT alone group -161 (IQR -210 to -455), CT+ZOL -13 (IQR -484 to +548) p=0.44).

At day 21 there was a significant difference in serum activin from baseline in ER-ve patients treated with CT compared to CT+ZOL. Activin significantly increased from baseline levels with the addition of zoledronic acid to chemotherapy in ER-ve patients (median change from baseline to day 21 pg/ml; CT alone 10.6 (IQR -38.3 to 53.9), CT+ZOL 80.9(IQR 60.8 to 106.7) p=0.0381), an effect not seen in patients with ER+ve tumours. This significant fall in serum follistatin with zoledronic acid at day 5 in ER-ve patients, was lost by day 21 (median change baseline to day 21 pg/ml; CT alone -62 (IQR -803 to 593), CT+ZOL -796 (IQR -1955 to -177) p=0.1714 (Table 4.8).

TGFβ1 was also differentially altered at day 21 compared to baseline according to ER status, with addition of zoledronic acid to chemotherapy in ER+ve patients increasing TGFβ1 at day 21, an effect not seen in ER-ve patients (median change baseline to day 21 pg/ml; CT alone 1828 (IQR -2710 to 7008), CT+ZOL 12259 (IQR 5346 to 17934) p=0.0057 (Table 4.8).

These results suggest that zoledronic acid may be altering levels of follistatin, activin and TGFβ1 differentially according to ER status of the primary tumour. Changes in the primary tumour secretion of these factors would be expected to occur early after administration of zoledronic acid +/- chemotherapy i.e. day 5, rather than at the day 21 time-point, suggesting that the changes seen in follistatin at day 5 may be linked to differential primary tumour secretion of follistatin according to ER status. However, the numbers per group are small and these results are exploratory.

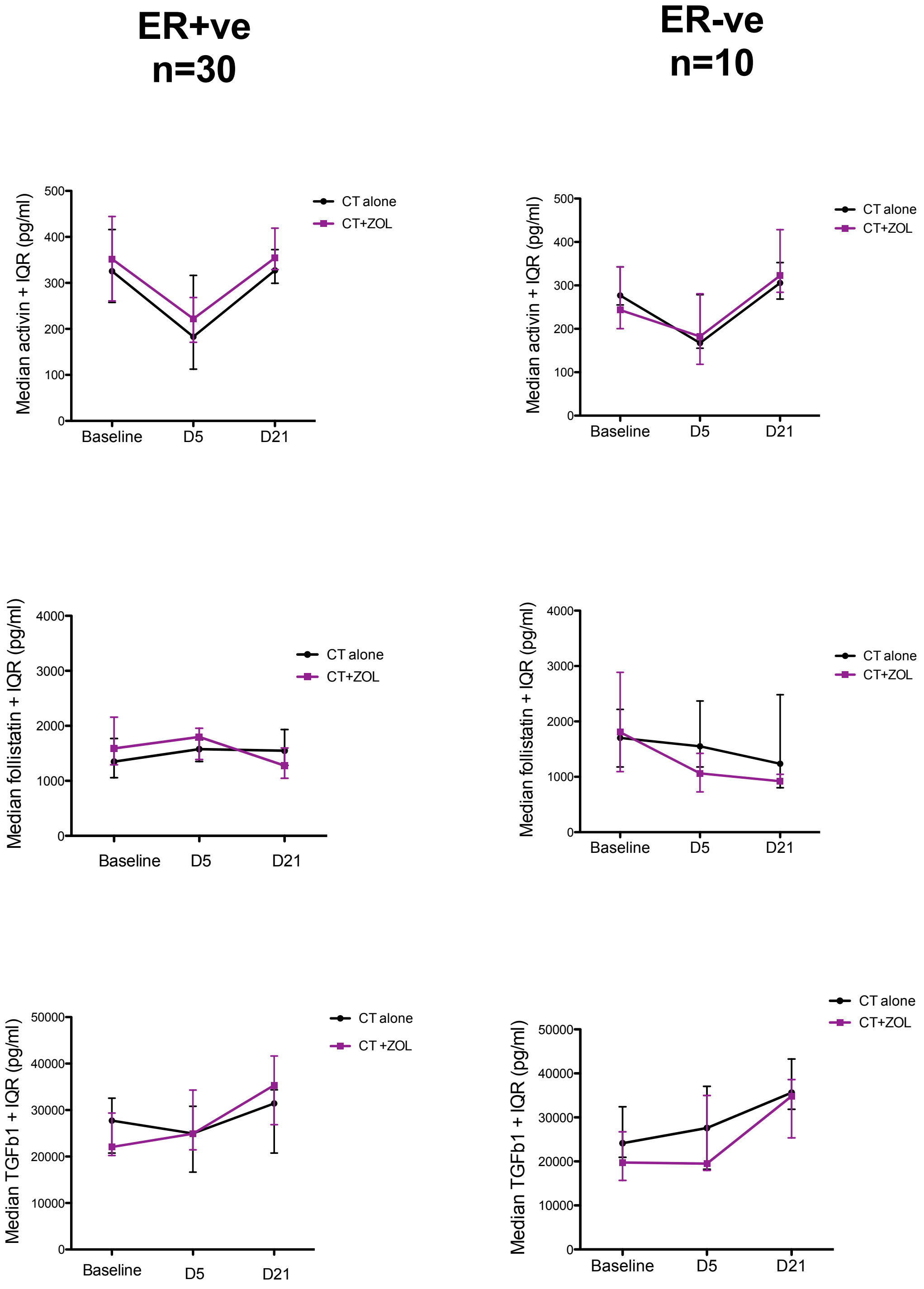


Figure 4.11 Changes in serum activin, follistatin and TGFβ1 according to oestrogen receptor (ER) status and treatment received.

Data represents median +IQR at each time-point in ER +ve patients (left) and ER-ve patients (right).

Table 4.8 Changes in activin, follistatin and TGFβ1 according to oestrogen receptor (ER) status of primary tumour.

Data represents median (IQR) change from baseline to day 5 (D5) and day 21 (D21). Mann Whitney test for significance between treatment groups. * p value <0.05

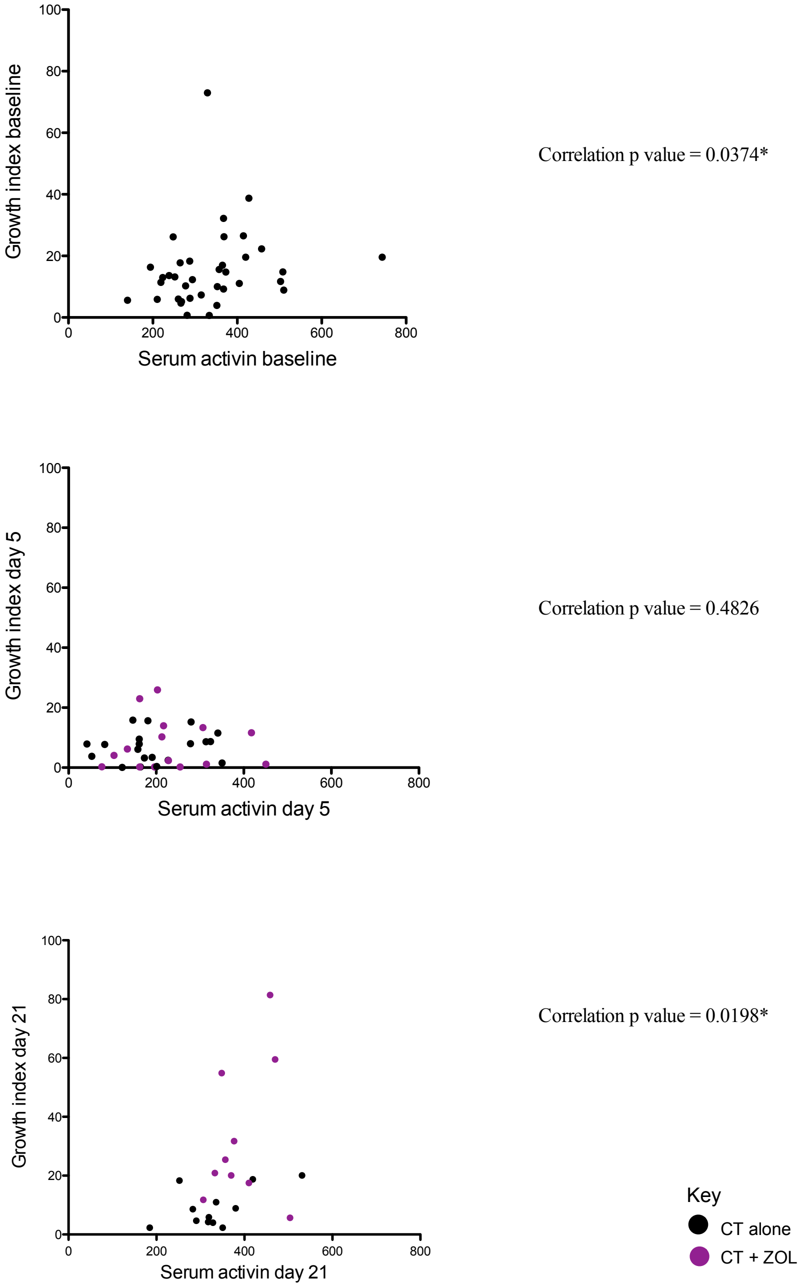
ER status and measured factor	Time-point	Treatment	Median Change pg/ml	IQR	P value
ER+ve Activin	**Baseline to D5**	**CT CT+ZOL**	**-135 -101**	**-213 to -88 -142 to -69**	**0.23**
	Baseline to D21	**CT CT+ZOL**	**-4.9 10.1**	**-67.8 to 30.3 -14.9 to 82.1**	**0.23**
ER-ve Activin	**Baseline to D5**	**CT CT+ZOL**	**-110 -98**	**-143 to -59 -149 to 43**	**0.91**
	Baseline to D21	**CT CT+ZOL**	**10.6 80.9**	**-38.3 to 53.9 60.8 to 106.7**	**0.038***
ER+ve Follistatin	**Baseline to D5**	**CT CT+ZOL**	**161 -13**	**-210 to -455 -484 to +584**	**0.44**
	Baseline to D21	**CT CT+ZOL**	**137 222**	**-134 to 421 -484 to 201**	**0.065**
ER-ve Follistatin	**Baseline to D5**	**CT CT+ZOL**	**156 -528**	**-123 to 440 -1787 to -257**	**0.009***
	Baseline to D21	**CT CT+ZOL**	**-62 -796**	**-803 to 593 -1955 to -177**	**0.171**
ER+ve TGFβ1	**Baseline to D5**	**CT CT+ZOL**	**-1716 3739**	**-4738 to +2106 -6187 to +10900**	**0.27**
	Baseline to D21	**CT CT+ZOL**	**1828 12259**	**-2710 to 7008 5346 to 17934**	**0.005***
ER-ve TGFβ1	**Baseline to D5**	**CT CT+ZOL**	**-1610 -1578**	**-10309 to +14856 -6771 to 18624**	**0.76**
	Baseline to D21	**CT CT+ZOL**	**10251 10221**	**4205 to 16765 4866 to 21520**	**1.0**

4.5.4 Correlation of serum levels of activin, follistatin and TGFβ to growth index of primary breast tumour biopsies.

These data have shown that zoledronic acid, when added to standard chemotherapy, has biological effects on serum levels of TGFβ1, follistatin and activin and this can be further influenced by menopausal status and ER status of the primary tumour. Serum measurements however, are unable to identify the source of secretion of these paracrine proteins. As part of the ANZAC trial, growth index (ki67/apoptosis) from repeat breast biopsies was measured by methods previously reported (Winter, Wilson *et al*. 2013).

Growth index results were available in 100% of patients at baseline, 88% (35/40) at day 5 and 55% (22/40) at day 21. Serum activin levels correlated with growth index at baseline (p=0.0374) and day 21 (p=0.0198) (Fig 4.12), and serum TGFβ1 levels correlated with growth index at baseline only (p=0.0029) (Fig 4.13). Serum follistatin levels did not correlate with growth index at any timepoint. Serum follistatin in ER-ve patients did not significantly correlate with growth index at day 5 either, however, growth index was only available in 9 patients at this time-point.

These data suggest, at baseline, activin and TGFβ1 may be secreted by the tumour cells, since higher serum levels correlate significantly with a higher growth index. After treatment only activin remains significantly correlated with growth index at day 21 suggesting that activin is secreted primarily by tumour cells compared to TGFβ1, which may be secreted from other sources. The source of follistatin may not be the primary tumour as there was no correlation with growth index, however, numbers in each group are small and these analyses are exploratory only.



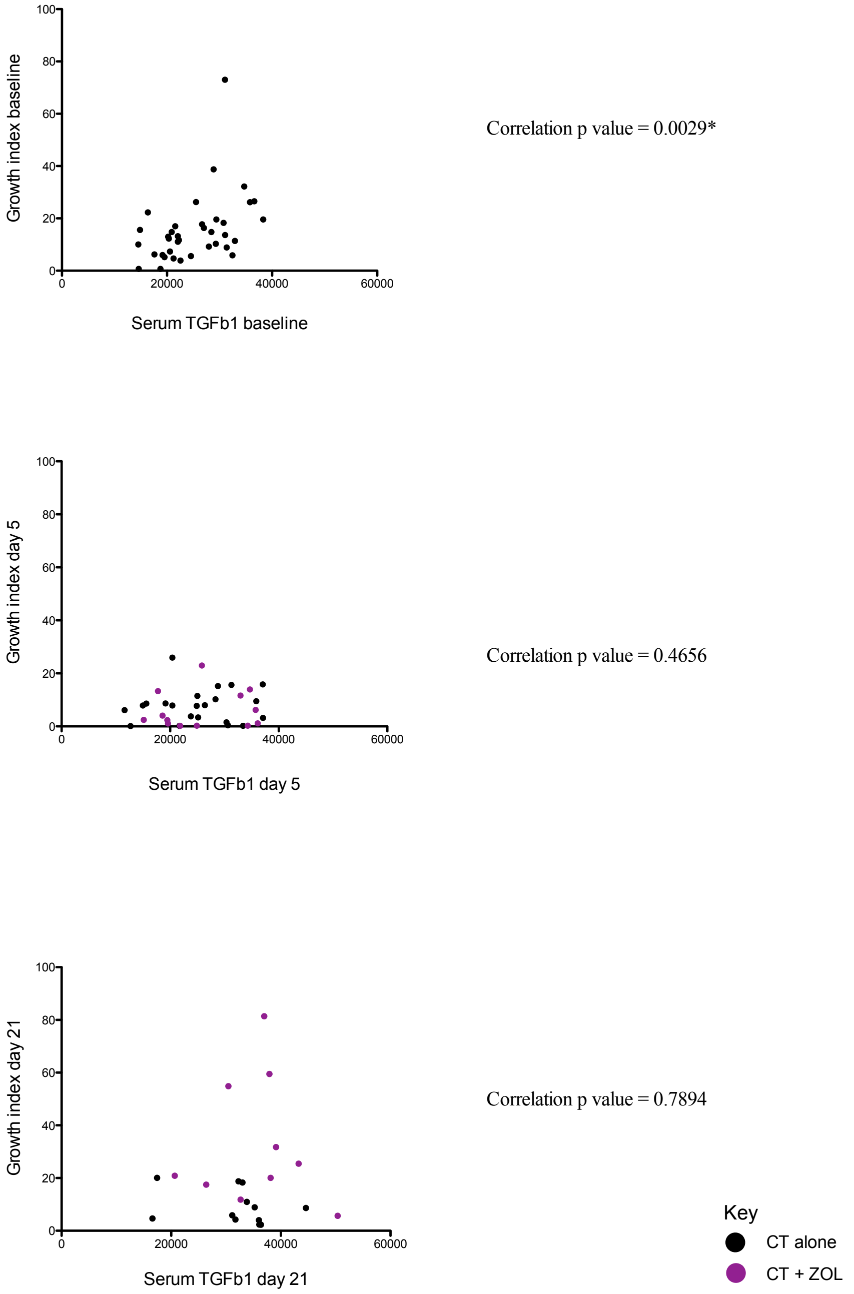
Correlation p value = 0.0374*

Correlation p value = 0.4826

Correlation p value = 0.0198*

Figure 4.12. Correlation of growth index with serum activin levels.

Data represents individual serum levels plotted against corresponding growth index from the overall population for baseline (top), day 5 (middle) and day 21 (bottom). Coloured dots identify different treatment groups. Spearman's non parametric correlation coefficient for significance, * p value <0.05



Correlation p value = 0.0029*

Correlation p value = 0.4656

Correlation p value = 0.7894

Figure 4.13. Correlation of growth index with serum TGFβ1 levels.

Data represents individual serum levels plotted against corresponding growth index from the overall population for baseline (top), day 5 (middle) and day 21 (bottom). Coloured dots identify different treatment groups. Spearman's non parametric correlation coefficient for significance,* p value <0.05

4.6 Discussion

These data are the first to show that neo-adjuvant zoledronic acid significantly affects serum levels of the TGFβ superfamily of proteins, and their inhibitors, in both a heterogeneous breast cancer population, and in specific subgroups according to menopausal status and ER status. At baseline, serum activin, follistatin and TGFβ are not affected by menopausal or ER status. However, baseline inhibin is significantly higher in premenopausal patients and declines significantly over time suggesting an effect of chemotherapy on ovarian function. After treatment with zoledronic acid, serum follistatin decreases at day 21 compared to baseline, with a concurrent increase in TGFβ in the overall study population. Evaluation of serum changes induced by zoledronic acid according to menopausal status and ER status showed a significant decrease in serum follistatin at day 21, and a concurrent increase in serum activin in postmenopausal women treated with zoledronic acid. This effect was not seen in premenopausal women. Postmenopausal women treated with neo-adjuvant zoledronic acid, would therefore be expected to have a higher bioavailability of the tumour suppressor activin, due to both an increase in activin and a decrease in the activin inhibitor follistatin, compared to premenopausal women treated with zoledronic acid. In addition, patients with ER-ve tumours showed a significant early fall in follistatin levels at day 5 with addition of zoledronic acid that is not seen in ER+ve patients. These novel effects of zoledronic acid on serum levels of the TGFβ superfamily may be relevant, since both postmenopausal and ER-ve patients have been shown in clinical trials to derive an increased anti-tumour effect from zoledronic acid.

Recent clinical evidence further supporting the neo-adjuvant anti-tumour efficacy of zoledronic acid in postmenopausal patients and patients with ER-ve tumours was presented by Horiguchi *et al* this year at the American Society of Clinical Oncology (ASCO) conference; 188 breast cancer patients aged 20-70 were randomised to receive zoledronic acid 4mg q3-4/52 for 7 treatments during neo-adjuvant FEC100 + weekly paclitaxel. There was a trend towards an improved pathological complete response in the ZOL+CT group vs CT (18.4% vs 7.8% p=0.16), and the difference between pCR rates was higher in postmenopausal patients (ZOL+CT 18.4%, CT 5.4%) and patients with receptor negative tumours (ZOL+CT 35.3%, CT 11.8%)(Horiguchi J 2013). This was further supported by the Neozotac study randomizing 250 patients to neo-adjuvant CT alone or CT+ZOL 4mg IV q3/52 during chemotherapy. Pathological results from 228 patients were presented this year at ASCO and showed no difference in the overall population in pCR rates, but a trend was observed to improved pCR in postmenopausal women (CT alone 18% va CT+ZOL 11%)(Charehbili A 2013).

The increased anti-tumour efficacy of zoledronic acid in these two subgroups of patients may be contributed to by the effects of zoledronic acid on the serum levels of TGFβ superfamily ligands, however, there are limitations in the ANZAC data which must be considered. The date of the last menstrual period (LMP) was not recorded for the pre menopausal patients, which made the interpretation of changes in inhibin over time difficult. However, the significant difference in serum inhibin between premenopausal women and postmenopausal women is in keeping with previously published data as discussed in chapter 3. The significant decline in inhibin in premenopausal women from baseline to day 21 may be due to a chemotherapy effect on ovarian function, or it may represent sampling of blood from premenopausal women at different time points in the menstrual cycle. During the menstrual cycle small antral follicles are recruited from the primordial follicle pool and progressively develop until only one is selected as the dominant follicle for ovulation. Inhibin A is highest in the late follicular phase of the menstrual cycle, reaching a peak at ovulation and then declining until a later surge 4-6 days post ovulation followed by a subsequent decline (de Kretser, Hedger *et al.* 2002). Inhibin is secreted by larger pre-ovulatory follicles in the follicular phase, and by the corpus luteum in the post ovulation surge (Hohmann, Laven *et al.* 2005). If, by chance, the majority of premenopausal patients at baseline were in the late follicular phase or 4-6 days post ovulation, then the inhibin A levels would have been higher at baseline, and 21 days later, there would be an expected cyclical decline in inhibin A. Alternatively, chemotherapy targets cycling cells and could therefore affect larger follicles that have been recruited into a phase of cell division and maturation. In a prospective study, inhibin A was measured in 30 breast cancer patient before chemotherapy, 4 weeks after completion of chemotherapy and at 2 years post chemotherapy. There was a significant decline in inhibin A at the completion of chemotherapy and 2 year time-point, compared to baseline (Burkhardt, Juckstock *et al.* 2010). There are no clinical studies in breast cancer to my knowledge that have evaluated the fall in inhibin A earlier than 6 months post initiation of chemotherapy. However, it has previously been shown that a single dose of CMF (cyclophosphamide 400mg/m^2, methotrexate 40mg/m^2, 5-fluorouracil 600mg/m^2

day 1 and 8) chemotherapy can increase the rate of amenorrhoea (defined as no menstrual bleeding for 3 consecutive months within 9 months of primary breast surgery) from 21% in women receiving no adjuvant chemotherapy to 31% in women receiving only 1 cycle of peri-operative CMF (Goldhirsch, Gelber *et al.* 1990). Therefore it would be conceivable that ovarian function could decline and serum inhibin may fall within 21 days of a single dose of $FEC_{100.}$

Whilst the major source of circulating inhibin is derived from ovarian secretion, it is less clear which sources contribute to circulating activin, follistatin and TGFβ1. ANZAC did not recruit non-tumour controls, and therefore interpretation of the changes in these paracrine proteins with zoledronic acid is challenging, as the serum alterations may be due to alterations in release of these proteins from tumour, bone or other sites.

Activin is abundant in bone matrix, and in the non-cancer setting has been shown to be elevated in the serum of postmenopausal patients (n=47) with a BMD T score <-2.0 lumbar spine, compared to postmenopausal controls (n=27) with normal bone density (BMD T score >-1.0 lumbar spine), suggesting activin A may have a negative role on the bone by increasing osteoclastogenesis (Anastasilakis, Polyzos *et al.* 2013). However, serum activin may increase as a consequence of increased bone turnover rather than the cause. In a cancer population, serum activin A was significantly higher in patients with breast cancer or prostate cancer (n=72) compared to sex and age matched controls (n-48), and was higher in breast and prostate patients with bone metastases compared to those without. In addition, in the breast cancer patients, the level of activin in serum correlated with the number of bone metastases (Leto, Incorvaia *et al.* 2006). Again it is unclear if this increase in serum activin with bone metastases compared to non-bone metastases is a cause or consequence of increased lytic bone destruction or increased tumour burden and thus secretion of activin. Interestingly, in the breast and prostate cancer patients without bone metastases, serum activin was elevated compared to healthy controls. This may imply the tumour cells are secreting enough activin in the local tumour environment to increase serum levels. This is supported by data from Reis *et al* (2002), who showed that activin levels were twice as high in breast tumour homogenates compared to non neoplastic tissue. In addition, in postmenopausal breast cancer patients undergoing surgery for removal of breast tumour, a significant decrease in serum activin A was seen at day 1 and 2 post-surgery compared to pre-surgery. A similar effect was not seen in women undergoing breast reduction surgery for non-malignant causes. Serum activin levels in the breast cancer patients, however, did not correlate with the presence of lymph nodes, tumour grade or tumour size (Reis, Cobellis *et al*. 2002). These data may suggest that activin secretion from tumour cells is a consequence rather than a cause of tumourigenesis since increased levels do not correlate with adverse prognostic factors. These data would support the correlation demonstrated between serum activin and growth index of the primary breast tumours at baseline and day 21 in the ANZAC study, suggesting that as the proliferation of breast tumours increases, a concurrent increase in serum activin occurs as a consequence of an increased number and activity of tumour cells.

Follistatin levels in serum are detectable in healthy subjects and are elevated in pregnancy and various pathological states such as liver disease and kidney disease (Sakamoto, Shintani *et al*. 1996). The main isoform of follistatin in the circulation is FS315, and follistatin binds activin in an almost irreversible complex that prevents activin interacting with its receptors (Sepporta, Tumminello *et al*. 2013). There is a paucity of data on follistatin levels in serum of breast cancer patients, but in prostate cancer patients (n=35), serum follistatin is significantly higher than patients with benign prostatic hypertrophy (n=20). In addition, serum follistatin correlated with the presence of bone metastases in the prostate cancer cohort (Tumminello, Badalamenti *et al*. 2010). These data suggest follistatin may be secreted by prostate cancer cells, or that follistatin may be either liberated from bone in the presence of bone metastases, or there is increased secretion from the prostate cancer cells when localized in bone. In the ANZAC serum cohort, follistatin did not correlate with growth index of primary tumour at any time-point, suggesting the source of follistatin may not be the primary breast cancer cells, but other sources such as bone.

TGFβ is abundantly stored in bone, and serum levels demonstrate a significant positive correlation with bone mineral density T score at lumbar vertebrae, radius and ulnar (Wu, Peng *et al*. 2013). In the breast cancer setting, TGFβ1 has been shown to be elevated in conditioned medium of breast cancer tumour explants compared to matched normal breast tissue (Toomey, Condron *et al*. 2001), and Dave *et al* found preoperative TGFβ1 to be elevated in the serum of patients with invasive breast cancer (n=117) compared to controls. These data suggest TGFβ1 is produced and released from the tumour in sufficient levels that can be detected in the circulation. In patients with advanced disease serum levels were significantly higher than early stage disease, suggesting levels may also correlate with tumour burden (Dave, Shah *et al*. 2012). Serum levels of TGFβ1 have also been shown to correlate with stages of early breast cancer. Sheen-Chen *et al* evaluated serum TGFβ1 from 60 patients with invasive breast cancer pre surgery, and compared them to 14 patients with benign breast tumours. They found that, although there was no significant difference between patients and controls, levels of TGFβ1 were significantly higher in patients with more advanced lymph node status, poorer histological grade and more advanced TNM (tumour, nodes, metastases) stage (Sheen-Chen, Chen *et al*. 2001). Similar positive correlation between serum TGFβ1 and lymph node involvement has also been reported in a cohort of 36 preoperative early breast cancer patients (Chod, Zavadova *et al*. 2008), again suggesting that serum TGFβ1 may be reflective of tumour burden. However, in a larger cohort of patients with invasive breast cancer (n=135) compared to DCIS (n=67), fibroadenoma (n=35) or healthy women 9 (n=40), no significant difference was found in serum levels between the groups, and in the invasive breast cancer patients TGFβ1 levels did not correlate with tumour stage, lymph node involvement or histological grade (Lebrecht, Grimm *et al*. 2004). Similar lack of difference in serum levels of 90 early stage breast cancer patients compared to 75 health controls was reported by Duranyildiz *et al* (Duranyildiz, Camlica *et al*. 2009). Taken together these data suggest that tumours do secrete TGFβ1, but it is not reliably differentially secreted in the circulation according to tumour characteristics or tumour burden to make it a good predictive or prognostic marker for breast cancer severity. The ANZAC data would support the correlation between baseline serum TGFβ1 and growth index of the primary tumours, suggesting that higher cell turnover secretes more TGFβ1 that can be detected in serum. However, in the ANZAC study, at day 21, serum TGFβ1 no longer correlated with growth index, implying that another source other than the primary tumour may be responsible for TGFβ1 secretion.

Considering these data it would seem plausible that the addition of zoledronic acid to standard chemotherapy could alter serum levels of these paracrine factors, since zoledronic acid has been demonstrated to affect both primary breast tumours (Clezardin, Ebetino *et al*. 2005) and bone resorption (Clezardin, Fournier *et al*. 2003). To my knowledge there are no other data from a breast cancer population that have evaluated changes in these factors with zoledronic acid treatment. The overall study population showed a decrease in serum follistatin and an increase in serum TGFβ1 with addition of zoledronic acid at day 21. Zoledronic acid has been shown to increase secretion of TGFβ1 from primary rat osteoblasts *in vitro* (Naidu, Dechow *et al*. 2008), suggesting that the effect of zoledronic acid on bone may potentially alter serum levels of TGFβ1. Osteoblastic *in vitro* cultures have shown that inhibitors of osteoblastic function i.e. dexamethasone, do so via an increase in follistatin mRNA expression, but pretreatment with the bisphosphonate alendronate can prevent this by reducing follistatin mRNA expression (Hayashi, Yamaguchi *et al*. 2009), indicating that bisphosphonates may effect follistatin expression in bone.

In the exploratory analyses of effects of zoledronic acid according to menopausal status and ER status, the decrease in follistatin at day 21 in postmenopausal patients was complemented by an increase in activin. In the non-cancer setting, a single infusion of zoledronic acid 5mg IV in postmenopausal women with low BMD does not alter serum activin at 3 months post infusion (Anastasilakis, Polyzos *et al*. 2013). This may indicate that the effect of zoledronic acid on serum activin in the ANZAC study was primarily due to direct tumour secretion of activin rather than from other sources such as bone, or may represent a different timing of serum collection (day 21 vs. 3 months).

The potential impact of these changes in serum levels of activin, follistatin and TGFβ1 can be hypothesized considering the known effects of these paracrine factors on breast cancer cell proliferation. As discussed earlier, preclinical data has show activin to be a suppressor of breast cancer cell proliferation, with follistatin inhibiting activin and its tumour suppressive effect. TGFβ1 can be both a tumor suppressor and a tumor promoter depending upon the stage of tumorigenesis. In the overall ANZAC population the significant decline in follistatin at day 21 with addition of zoledronic acid may represent a new and novel anti-tumor mechanism of zoledronic acid. By decreasing serum follistatin, zoledronic acid increases the bioavailability of the tumour suppressor activin. The significant increase in TGFβ1 at day 21 in the zoledronic acid group may have a tumour suppressive or promoter action on the primary breast tumor. It is interesting that the significant decline in serum follistatin with zoledronic acid at day 21 is driven by the postmenopausal cohort, which also demonstrate a significant increase in serum activin at day 21. Thus zoledronic acid may be having an even greater anti-tumour effect in postmenopausal women by both increasing activin and concurrently decreasing follistatin levels in serum. In patients with ER-ve tumours, an early significant fall in follistatin with zoledronic acid at day 5 with a significant increase in activin at day 21, may be contributing to the enhanced anti-tumour activity of zoledronic acid seen in neoadjuvant clinical trials in these patients.

It is not possible however, to extrapolate serum levels of these proteins to levels found within primary tumours, and therefore these conclusions and hypotheses are theoretical only. The possible enhanced anti-tumour activity of zoledronic acid via manipulation of the activin: follistatin system in postmenopausal women and patients with ER-ve tumours is novel and requires further investigation.

Chapter 5. Effects of zoledronic acid on the activin-signaling pathway in human breast cancer cell lines.

5.1 Summary

Neo-adjuvant breast cancer clinical trials have demonstrated that the anti-tumour efficacy of zoledronic acid is influenced by oestrogen receptor (ER) status, with ER-ve tumours being more responsive to the beneficial effects of zoledronic acid on disease free, overall survival and pathological complete response. The mechanism behind this differential effect remains to be identified. I have previously shown that zoledronic acid, when added to first cycle of neo-adjuvant chemotherapy, decreased serum levels of follistatin, which neutralizes the tumour suppressor activin, in patients with ER-ve tumours at day 5-post treatment, an effect not seen in patients with ER+ve tumours. It is unknown if zoledronic acid can directly affect the activin pathway in breast cancer cells, either at cell surface receptor level, or by influencing downstream signaling pathways. The aims of the work in this chapter was therefore to evaluate the effect of zoledronic acid on activin and follistatin in ER-ve and ER+ve breast cancer cell lines *in vitro*.

Zoledronic acid decreased the paracrine secretion of the activin neutraliser, follistatin, in ER-ve breast cancer cell lines only (MDA-MB-231 and MDA-MB-436). The functional implication of this was investigated using cell proliferation assays in ER-ve and ER+ve cell lines to assess the effect of recombinant activin, +/- follistatin. Follistatin negated the anti-proliferative effect of activin in MDA-MB-231 and MCF7 cells. In addition if the activin type I receptor (ALK4/5) was blocked using the inhibitor SB-431542, the anti-proliferative effect of activin was decreased in MDA-MB-231 cells, indicating blocking activin either at receptor level or outside the tumour cells, prevents it acting as a tumour supressor. The effects of zoledronic acid on the signaling pathways downstream of the ALK4 receptor were assessed by focusing on Smad2, and its two main phosphorylation sites using immunofluorescence. Zoledronic acid prevented nuclear localisation of the tumour promoting linker-phosphorylated Smad2 in MDA-MB-231 cells, and increased the levels of the tumour suppressor c terminus-phosphorylated Smad2.

These *in vitro* findings were confirmed in a sub-cutaneous xenograft model of ER-ve MDA-MB-436 tumours treated with zoledronic acid, which showed a decrease in the area of tumour stained +ve for follistatin, and a decrease in the number of cells stained +ve for linker phosphorylated-Smad2 compared to saline control.

These data are the first to demonstrate zoledronic acid can affect the activin-signaling pathway in ER-ve breast cancer cells both *in vitro* and *in vivo.*

5.2 Introduction

Neo-adjuvant chemotherapy is used in early breast cancer to change inoperable breast tumours to operable, or to enable breast-conserving surgery to be performed. The addition of zoledronic acid to neo-adjuvant chemotherapy has been shown to significantly reduce the residual invasive tumour size compared to chemotherapy alone (Coleman, Winter *et al*. 2010), suggesting a direct anti-tumour effect of zoledronic acid on both ER-ve and ER+ve breast tumours. However, not all breast tumours are equally responsive to zoledronic acid, and clinical studies have shown that addition of zoledronic acid to neo-adjuvant chemotherapy reduces disease recurrence and death in patients with ER-ve, but not ER+ve, tumours (Aft, Naughton *et al*. 2012) (Horiguchi J 2013). The molecular mechanism underpinning this differential effect of zoledronic acid according to ER status has not yet been identified. *In vitro,* zoledronic acid inhibits proliferation of the ER-ve cell line MDA-MB-231 and induces apoptosis via an increase in the levels of pro-apoptotic factors. This effect of zoledronic acid was not seen in the ER+ve cell line MCF7 (Rachner, Singh *et al*. 2010). An ER dependent anti-proliferative effect of the bisphosphonate clodronate has also been reported, with a reduction in tumour cell survival of the ER-ve MDA-MB-435S cell line, but no effect was shown in MCF7 cells (Busch, Rave-Frank *et al*. 1998). The *in vitro* anti-tumour affects of zoledronic acid include reduced survival, adhesion, migration and invasion of tumour cells, and are thought to be due to the effect on the mevalonate pathway, via inhibition of FPP synthase and reduced prenylation of small GTPases (Gnant and Clezardin 2012). Whether the differential anti-tumour affect of zoledronic acid according to ER status is due to an effect on the mevalonate pathway is not known.

The neo-adjuvant ANZAC study (n=40) showed a menopause (Winter, Wilson *et al*. 2013) and tumour ER status (Wilson C 2013) dependent effect on serum levels of follistatin following administration of zoledronic acid. At day 5 post FEC_{100} chemotherapy, in patients with ER-ve tumours, the addition of 4mg IV zoledronic acid resulted in a significantly lower serum level of follistatin compared to patients receiving chemotherapy alone. This may represent a fall in the secretion of follistatin from ER-ve breast tumours that are not seen in ER+ve tumours. Follistatin is a paracrine antagonist of activin and both proteins modify breast cancer cell proliferation. Activin is produced by breast cancer cells and inhibits their proliferation; follistatin binds to activin and prevents receptor binding with the type II receptor (ActRII), thus promoting proliferation (Bloise, Couto *et al*. 2009). Once activin binds to ActRII, dimerization occurs with ActRIB (ALK4) and the receptor associated intracellular proteins, Smad2 and 3, are phosphorylated. They subsequently translocate to the nucleus with the co-receptor Smad4 (Liu and Feng 2010) (Chapter 1 Fig 1.13). Smads undergo phosphorylation at the COOH-tail following receptor dimerization, or at a linker region joining the MH1 and MH2 domains by intracellular protein kinases primarily RAS and cyclin dependent kinases (Matsuzaki 2011). These different phosphorylation sites have different effector functions; the COOH-tail being a tumour suppressor and the linker phosphorylation site being a tumour promoter (Matsuzaki 2011). Previously published *in vitro* data suggested ER-ve cell lines were resistant to the anti-proliferative effects of activin as a result of low expression of the activin type II receptor, however, MDA-MB-231 were found to express sufficient type II receptors suggesting an alternative mechanism of resistance to activin (Kalkhoven, Roelen *et al*. 1995). Further *in vitro* studies found the ER-ve cell line MDA-MB-436 to have a functional activin-signaling pathway by demonstration of phosphorylation of Smad2 in response to exogenous activin, and proliferation was inhibited by exogenous activin following removal of follistatin from the medium or silencing of the follistatin related gene (FLRG)(Razanajaona, Joguet *et al*. 2007). These data indicate that exogenous neutralisers of activin, such as follistatin, are responsible for the lack of inhibition of proliferation in response to activin in ER-ve cell lines, rather than lack of/non functional activin receptors.

To my knowledge there are no *in vitro* studies evaluating the ability of zoledronic acid to affect activin and follistatin in breast cancer cell lines. The new information in this chapter details how zoledronic acid can affect the activin signaling pathway by a dual mechanism in ER-ve breast cancer cell lines; **decreasing secretion of follistatin** and **altering the localization of linker phosphorylated Smad2**. These data suggest a potential novel mechanism to explain the differential tumour effect of zoledronic acid according to ER status.

5.3 Aims

The overall purpose of this chapter was to evaluate the affect of zoledronic acid on the activin-signaling pathway in breast cancer cells *in vitro* and *in vivo*. The following aims were set;

1. Evaluation of activin, follistatin and TGFβ secretion from ER-ve and ER+ve breast cancer cell lines *in vitro.*

2. Determine the effect of zoledronic acid on secretion of activin and follistatin from ER-ve and ER+ve breast cancer cell lines *in vitro.*

3. Establish the functional effect of activin and follistatin on proliferation of ER-ve and ER+ve cell lines.

4. Evaluate the effect of zoledronic acid on the downstream activin-signaling pathways, focusing on COOH-tail phosphorylation and linker phosphorylation of Smad2 *in vitro.*

5. Assess the effect of zoledronic acid on follistatin and linker phosphorylated Smad2 levels in a xenograft model of sub-cutaneous ER-ve MDA-MB-436 tumours.

5.4 Materials and methods

The following section contains a brief description of the methods used for the studies included in this chapter. See materials and methods chapter 2 for further information.

5.4.1 Secretion of activin and follistatin from ER-ve and ER+ve cell lines.

Two ER-ve cell lines were used (MDA-MB-231 and MDA-MB-436) and two ER+ve cell lines (MCF7 and T47D). Cells were seeded in 6 well plates at densities chosen to reflect specific cell line rate of replication, with an aim to achieve ~80% confluence of cells at the end of the experiment. MDA-MB-231 and MCF7 cells were maintained in DMEM+10%FCS for 24hrs and 48 hrs with supernatant removed at each time-point and frozen for ELISA. Live cell count was performed from each well at the end of the experiment, to enable normalization of activin/follistatin levels to cell count by expressing results as secreted concentration/million cells. MDA-MB-436 and T47D cells were maintained for 48hrs.

5.4.2 Effect of zoledronic acid on the secretion of activin and follistatin from ER-ve and ER+ve breast cancer cell lines, and effect on proliferation.

MDA-MB-231, MBA-MB-436, MCF7 and T47D cell lines were seeded in 6 well plates at densities previously described. Cells were exposed to 50μM zoledronic acid for 48-hours. At the end of the experiment supernatant was collected for ELISA and live cell count performed as previously described to enable normalization of activin/follistatin levels to cell number. The experiment was repeated in the ER-ve cell lines using a 4-hour pulse of zoledronic acid, followed by removal of drug and replacement with medium for a further 44 hours.

5.4.3 Effect of activin and follistatin on proliferation of ER-ve and ER+ve cell lines.

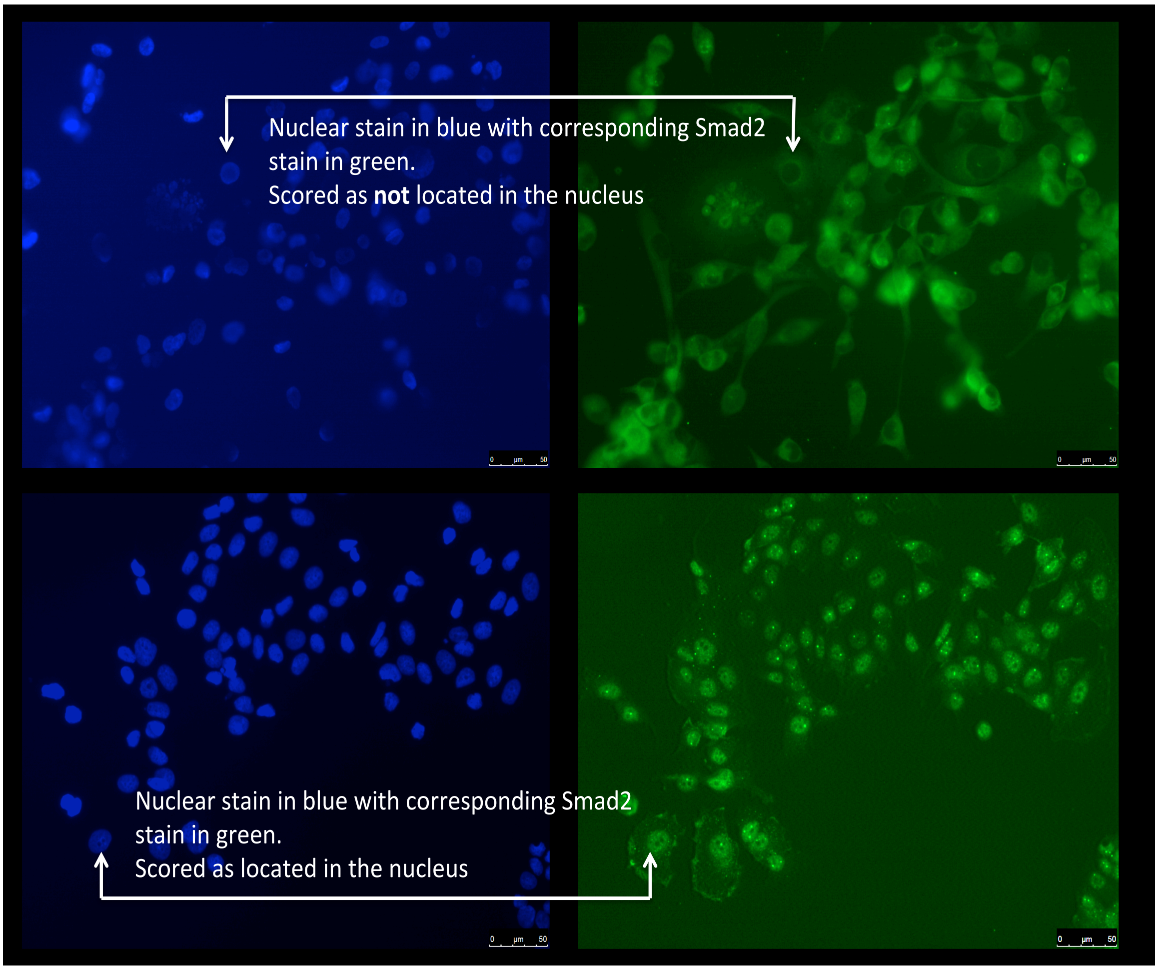
MDA-MB-231 and MCF7 cells were seeded in 96 well plates at densities detailed in chapter 2. Activin dissolved in RPMI+10%FCS was added to each well on day 0 at concentrations ranging from 60pg/ml to 6000pg/ml. The wells were washed and medium +/- activin was changed every 24 hours due to the predicted endogenous secretion of the activin antagonist follistatin from the cell lines. Plates were developed at day 1,3 and 5 by addition of a compound that degrades to a coloured formazan product that can be quantified using absorbance on a plate reader. Based on the results of the activin proliferation assays, the optimal time-point was determined as day 3 for further proliferation assays using activin in combination with follistatin and activin in combination with an ALK4/5 inhibitor. All proliferation experiments were carried out with 8 replicates and 3 repeats.

5.4.4 Effect of zoledronic acid on downstream activin-signaling pathways.

MDA-MB-231 and MCF7 cells were seeded in chamber slides, as described in chapter 2, containing RPMI +10%FCS. A 48-hour endpoint was maintained in these experiments based on previous results. After fixation of cells at the end of the experiment, one chamber was maintained as the –ve control with addition of 5%BSA only, and primary antibody in 5% BSA was added to the other 7 chambers. Primary antibodies used were to detect phosphorylated COOH-tail Smad2 (serine 465/467) or phosphorylated linker Smad2 (serine 245/250/255). Chamber slides were scored using a Leica DMI 400B inverted fluorescent microscope. Nuclear localization was identified using paired images of DAPI stained nuclei with fluorescent stained Smad2, and manually quantified from saved images taken from 4 quadrants of each chamber and expressed as a percentage of total number of cells (Fig 5.1). To quantify the level of phosphorylated linker Smad2 (pSmad2L) western blot was used. To quantify the level of phosphorylated COOH-tail Smad2 (pSmad2C) relative to total Smad2 a cell-based immunoassay was used which utilized 2 different fluorescent secondary antibodies to determine levels of each protein (see Chapter 2 Fig 2.2). MDA-MB-231 cells were plated and left for 48 hours in RPMI+10%FCS. Medium was removed and exchanged for serum free medium for 24 hours. Cell were then treated for 1 hour with supernatant from MDA-MB-231s previously treated with medium alone (control) or medium + 50μM zoledronic acid for 48 hours. Cell were then fixed and assay completed as per protocol.

5.4.5 Effect of zoledronic acid on follistatin and linker phosphorylated Smad2 in a sub-cutaneous xenograft model of ER-ve MDA-MB-436 tumours.

The in vivo component of this work was carried out by Dr Penny Ottewell and has been previously published (Ottewell, Monkkonen *et al*. 2008). To summarise the experimental



Nuclear stain in blue with corresponding Smad2 stain in green.
Scored as **not** located in the nucleus

Nuclear stain in blue with corresponding Smad2 stain in green.
Scored as located in the nucleus

Figure 5.1. Representative immunofluorescent images of tumour cells evaluated for cellular localization of phosphorylated Smad2 protein.

Blue DAPI nuclear stain was used with a paired green fluorescent pSmad2 stain to count the number of tumour cells with nuclear pSmad2, represented as a percentage of the total cells per image. Arrows indicate corresponding DAPI and pSmad2 images from cells counted as pSmad2 not located in the nucleus (top) and cells counted as pSmad2 located in the nucleus (bottom) outline, female MF1 nude mice were injected sub-cutaneous with $5x10^5$ MDA-MB-436 cells, and weekly zoledronic acid 100µg/kg intra peritoneal vs. saline control was given from day 7. Mice were maintained for 6 weeks then culled 24 hours after the last zoledronic acid injection (Fig 5.2). Tumours were isolated and fixed in 4% paraformaldehyde in PBS for 24 hours and subsequently paraffin embedded for IHC. Sections were cut and stained for follistatin or linker phosphorylated Smad2 using methodology described in chapter 2. Two sections per tumour were stained with each primary antibody and 20 x $750µm^2$ images were scored from each tumour using an Leica BMRB upright microscope and Osteomeasure software (OsteoMetrics Inc., Decatur, GA). Follistatin was scored according to the intensity of staining graded as 0 no staining, 1 weak staining, 2 moderate staining and 3 strong staining, and the area of staining as 0 no staining, 1 up to 10% of the tumour area, 2 10-50% of the tumor area, 3 >50% of the tumour area. pSmad2L was scored by counting the number of positively stained cells per tumour area (Fig 5.3).

5.4.6 Statistical analysis

Unless stated otherwise, all experiments were carried out with 3 replicates and 3 repeats. Prism GraphPad (5.0a) was used for statistical analysis. Data analysis was by non-parametric Mann-Whitney or Kruskal-Wallis with post hoc Dunns test. Data represent mean and SEM. Significance is defined as a p value <0.05 and represented graphically by * or p value <0.01 ** or P value <0.001 ***.

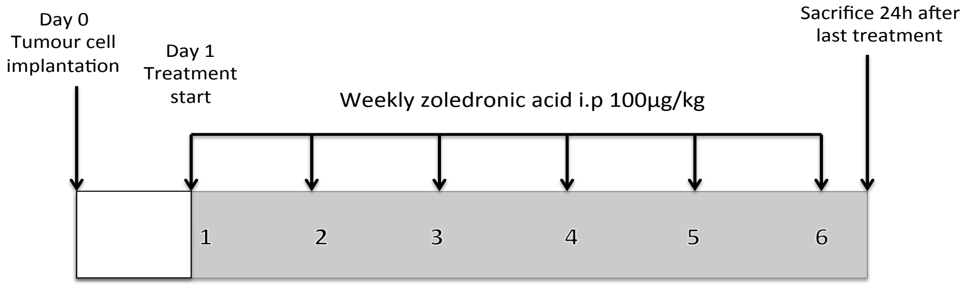


Figure 5.2. Experimental outline for xenograft model of sub-cutaneous MDA-MB-436 tumours.

Female MF1 nude mice (n=8-9 per group) were injected with $5x10^5$ MDA-MB-436 tumour cells in right flank and treatment commenced with weekly zoledronic acid at day 7 when tumours were palpable. Mice were culled 24 hours after the final zoledronic acid treatment.

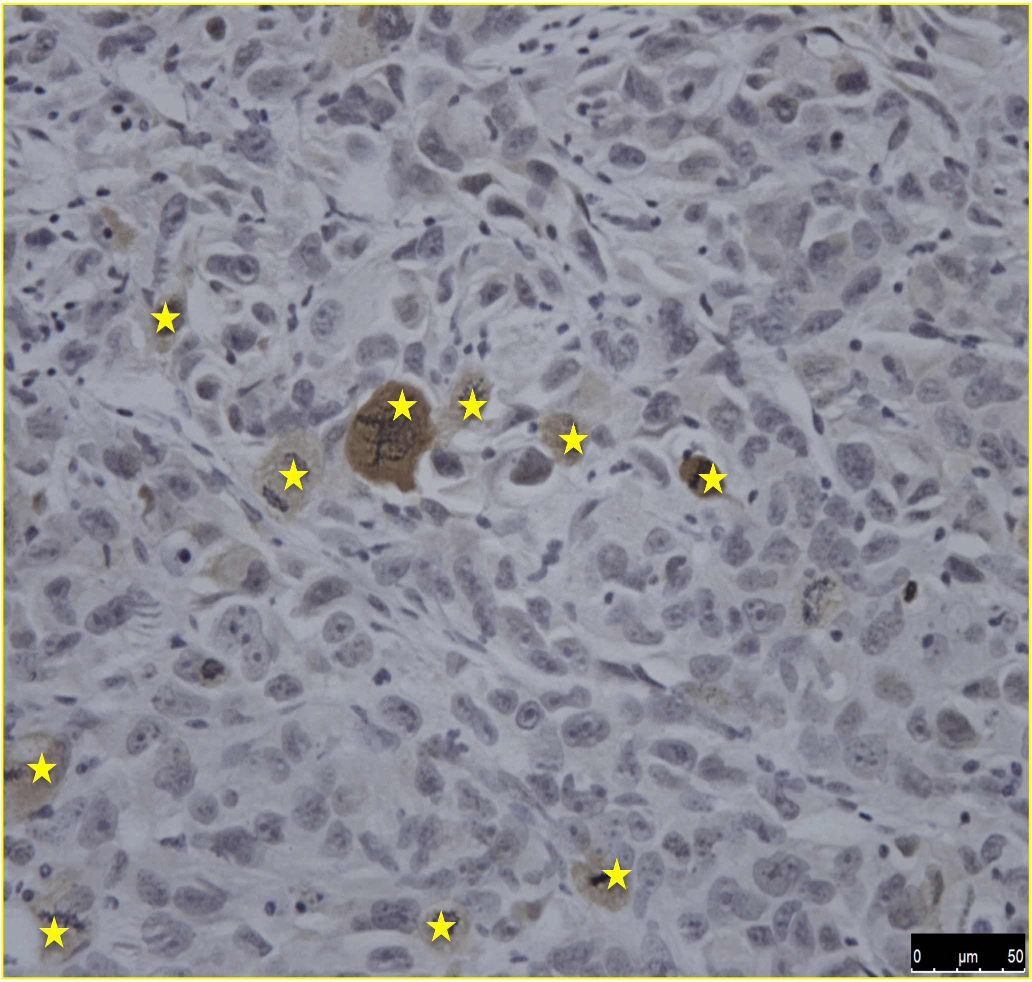


Figure 5.3. Representative image of sub-cutaneous MDA-MB-436 tumour evaluated for expression of pSmad2L.

Cells marked with a yellow star were counted as being positive for pSmad2L due to the intensity of their positive DAB stain (brown). 20x 750µm^2 images were scored to give an average score per tumour.

5.5 Results

5.5.1 Secretion of activin and follistatin from ER-ve and ER+ve cell lines.

To determine if breast cancer cell lines secrete activin, follistatin and TGFβ1 *in vitro,* ELISAs specific to human activin, follistatin and TGFβ1 were used to analyse cell culture supernatant collected from 4 different breast cancer cell lines. There was no significant secretion of TGFβ1 at 48 hours, however, all cell lines secreted both activin and follistatin in cell culture at 48 hours (Fig 5.4). Both ER-ve cell lines secreted significant levels of activin at 48 hours compared to medium alone (MDA-MB-231 p value= 0.0034, MDA-MB-436 p value= 0.0436). The ER-ve cell line MDA-MB 231 secreted significant levels of follistatin at 48 hours compared to medium alone (p value=0.0088). In ER+ve cell lines follistatin and activin secretion was not significantly increased at 48 hours. This time-point was used in subsequent experiments assessing changes in secreted activin and follistatin with addition of zoledronic acid.

5.5.2 Effect of zoledronic acid on the secretion of activin and follistatin from ER-ve and ER+ve breast cancer cell lines, and effect on proliferation.

To determine if zoledronic acid can alter the secretion of either activin or follistatin from MDA-MB-231 and MCF7 cells, both cell lines were exposed to medium alone or medium containing 25μM or 50μM zol for 48 hours. Cell supernatant was collected and evaluated for levels of activin and follistatin measured by ELISA with values normalized to live cell count at the end of the experiment. This was to ensure an accurate reflection of the quantity of soluble protein secreted relative to cell number, because 48 hours of continuous exposure to zoledronic acid (50μM) significantly lowered live cell count in MDA-MB-231 and MDA-MB-436 cells compared to control (MDA-MB-231 p value =0.0009, MDA-MB-436 p value =0.0045) but not MCF7 or T47D cells (Fig 5.5). 25μM zoledronic acid did not cause a significant change in secretion of activin or follistatin secretion with in any cell lines. Follistatin secretion from MDA-MB-231 cells was significantly decreased after exposure to 50μM zoledronic acid compared to control (p value =0.0012) (Fig 5.6). This effect was not seen in the MCF7 cells.

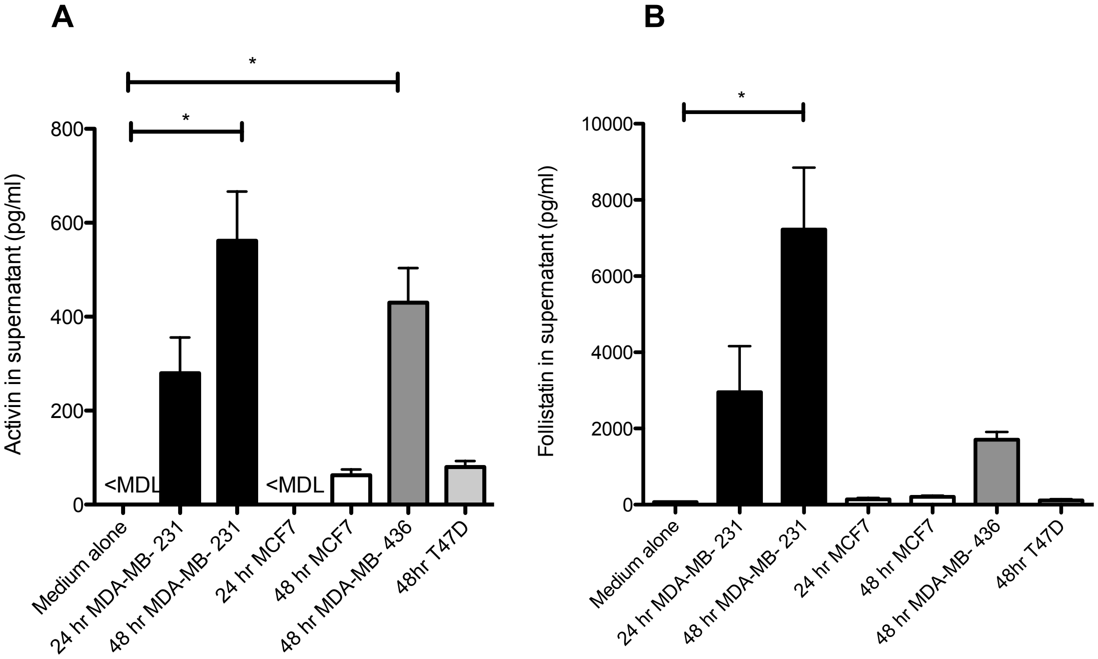


Figure 5.4. Activin and follistatin secretion from ER- breast cancer cell lines (MDA-MB-231 and MDA-MB-436) and ER+ve breast cancer cell lines (MCF7 and T47D).

The cell lines were plated in 6 well plates. Activin (**A**) and follistatin (**B**) secretion into supernatant was determined by ELISA at 24 and 48 hours. Data represents 3 replicates and 3 repeats. Mann Whitney test for significance comparing wells with cells to media alone (no cells), *p value <0.05. <MDL = below assay minimum detection limit.

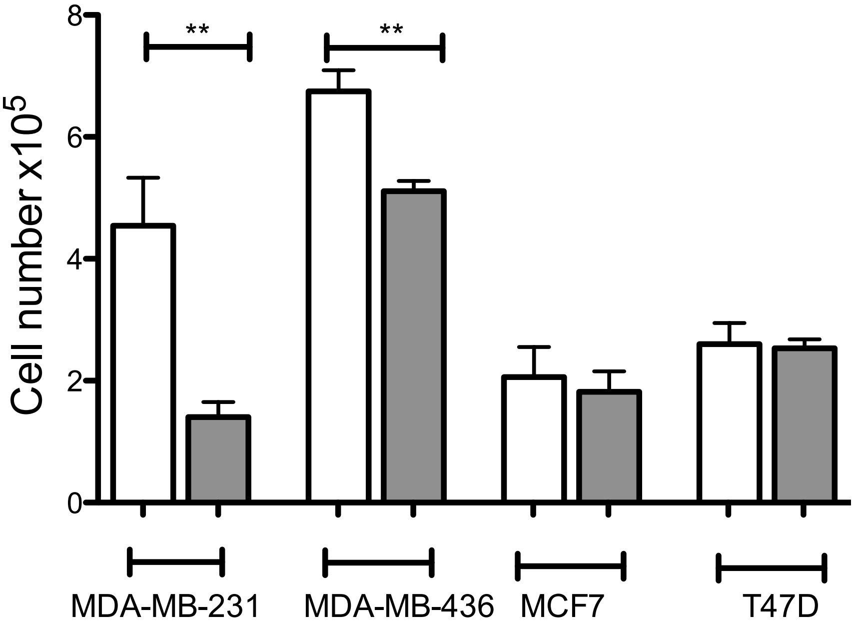


Figure 5.5. Effect of 48 hours of treatment with 50µM zoledronic acid on cell viability.

ER-ve MDA-MB-231 and MDA-MB-436 cells and ER+ve MCF7 and T47D cells were plated in 6 well plates and maintained in medium alone (open bars) +/- 50µM ZOL (closed bars). Data represents 3 replicates and 3 repeats. Mann-Whitney test for comparison of control with ZOL treated, **p value <0.01.

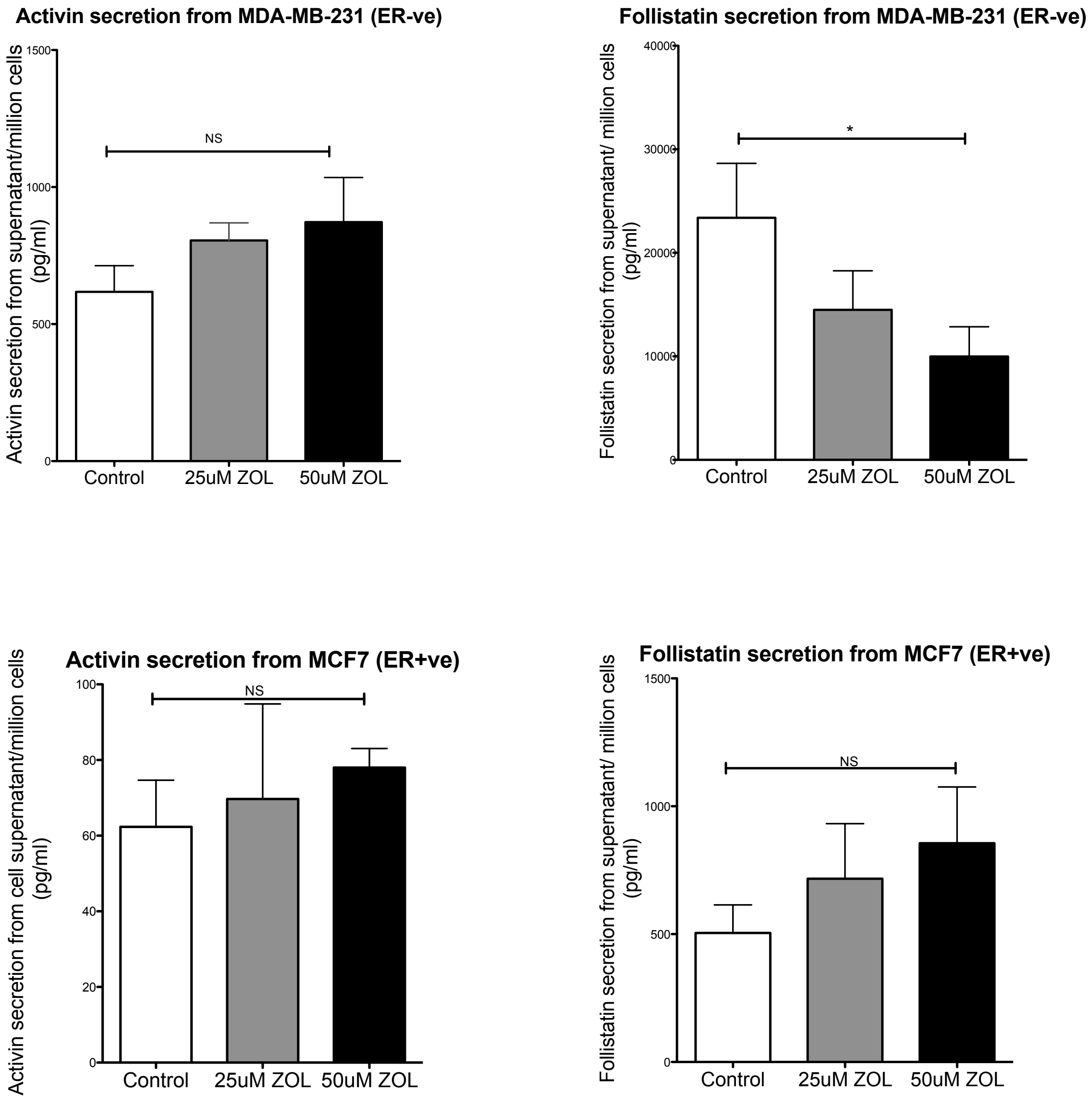


Figure 5.6. Activin and follistatin secretion from MDA-MB-231 (ER-ve) and MCF7 (ER+ve) cells in response to 48 hours exposure to increasing doses of zoledronic acid.

Cells were plated in 6 well plates and treated with medium alone or with addition of 25μM or 50μM ZOL. Data represents 3 replicates and 3 repeats. Mann Whitney test for significance comparing each dose to control, * p value <0.05, NS= not significant.

Activin secretion was not significantly altered after exposure to 50μM Zoledronic acid in either cell line. Since the 'biological activity' of activin will be relative to follistatin levels the molar ratio of follistatin:activin was calculated. The literature reports a 4:1 ratio of follistatin:activin would neutralize activin (Harrison, Chan *et al*. 2006). The mean concentrations of secreted follistatin and activin from each treatment group were converted into pmol/L, by dividing by the relevant molecular weight, and expressed as a ratio (Fig 5.7). Addition of 50 μM zoledronic acid reduces the molar ratio of follistatin:activin in MDA-MB-231 cells (control ratio 14:1, ZOL ratio 4:1), compared to MCF7 cells which show a rise in the molar ratio of follistatin:activin with addition of zoledronic acid (control ratio 3:1, ZOL ratio 4:1).

Continuous treatment of breast cancer cells with zoledronic acid for 48 hours does not represent a clinically relevant duration of drug exposure for primary breast cancer cells. Zoledronic acid reaches a peak in the circulation at the end of the IV administration and declines to 10% of the peak level after 4 hours (Ltd 2013), therefore breast cancer cells located outside of bone will only be exposed to a short pulse of drug every 3-4 weeks. To evaluate the effects of a short exposure of zoledronic acid on the secretion of follistatin in ER-ve cell lines, two ER-ve cell lines (MDA-MB-231 and MDA-MB-436) were treated with 50μM zoledronic acid for 4 hours followed by 44 hours of medium alone and supernatant was evaluated for follistatin levels. Both cell lines significantly decreased their secretion of follistatin in response to a 4-hour pulse of zoledronic acid compared to control (MDA-MB-231 p value =0.0015, MDA-MB-436 p value = 0.001) (Fig 5.8).

These data demonstrate that even a relatively brief exposure to zoledronic acid decreased the secretion of follistatin from ER-ve cell lines. The lack of effect of zoledronic acid on both cell count and secretion of activin or follistatin in ER+ve cell lines suggested they may not take up zoledronic acid. To evaluate if ER+ve cell lines were able to take up the drug, western blot was used to evaluate if zoledronic acid can increase intracellular levels of unprenylated Rap1a in MCF7 cells, since zoledronic acid has been shown to increase accumulation of unprenylated small GTPases via inhibition of the mevalonate pathway

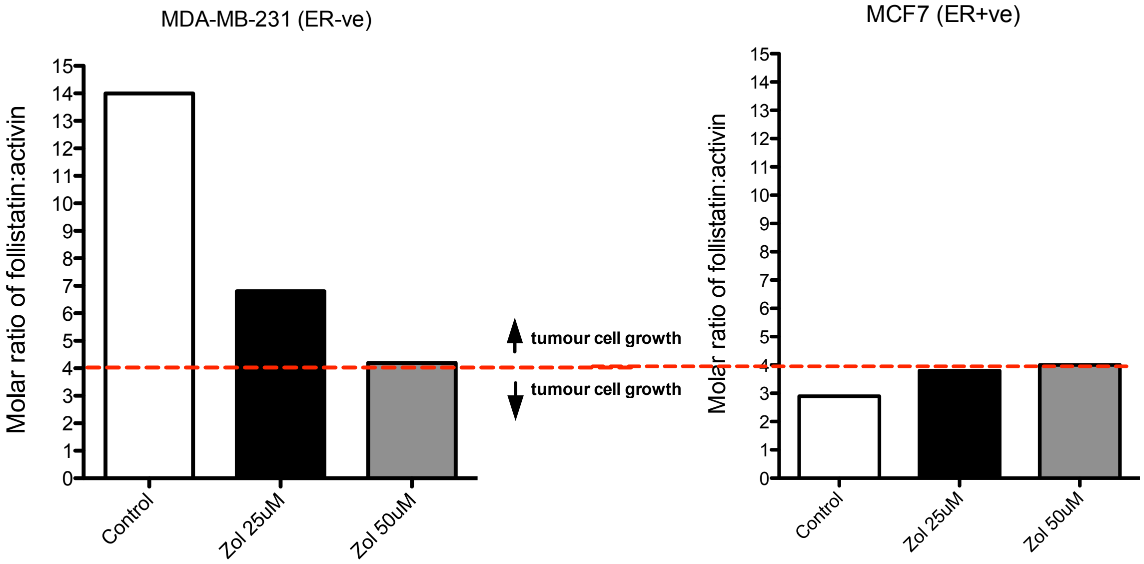


Figure 5.7 Molar ratio of follistatin:activin in MDA-MB-231 and MCF7 cells treated with increasing doses of zoledronic acid for 48 hours.

The mean quantity of secreted protein per million cells (pg/ml) was converted into pmol/ml for both activin and follistatin by dividing by their molecular weight. The molar ratio was then calculated by dividing follistatin by activin. The red horizontal lines represent the molar ratio of follistatin: activin that neutralizes activin, bars above the red line represent an excess molar ratio of follistatin to activin, bars below the red line represent an excess molar ratio of activin to follistatin.

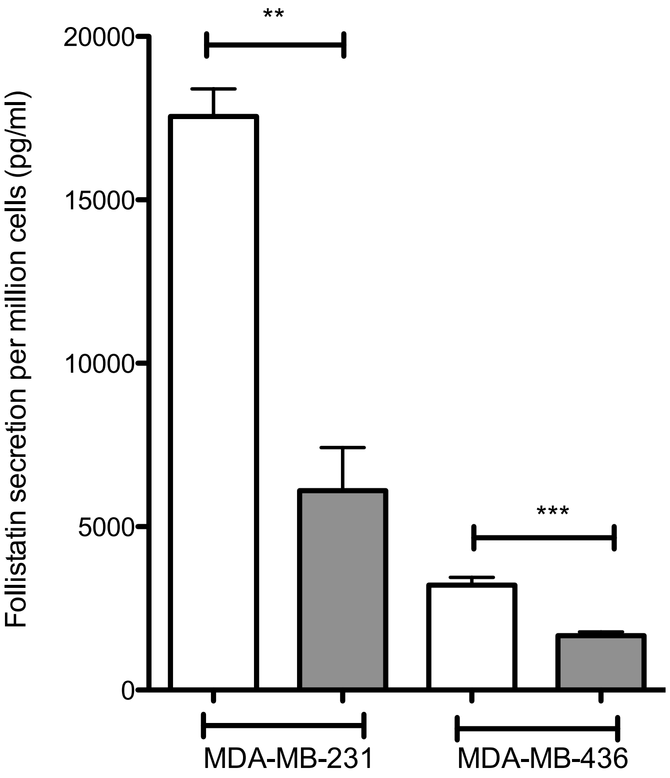


Figure 5.8. Changes in follistatin secretion from ER-ve cell lines in response to a 4 hour pulse of 50μM zoledronic acid.

Both cell lines were plated in 6 well plates in the presence of medium alone (open bars) or medium + 50μM zoledronic acid (closed bars) for 4 hours. Cells were then washed with PBS and medium was added to all cells for a following 44 hours. Data represent 3 replicates and 3 repeats. Mann Whitney test for significance comparing ZOL treated cells to their respective control, ** p value <0.005, *** p value <0.001.

(Russell 2011). In addition, by adding the mevalonate pathway intermediary, geranylgeraniol (GGOH), the effects of zoledronic acid on accumulation of unprenylated Rap1a should reverse. Both MDA-MB-231 and MCF7 cell lines showed an increase in the accumulation of unprenylated Rap1a in response to treatment with zoledronic acid. Addition of GGOH partly reversed the effects of zoledronic acid (as shown by a decease in unprenylated Rap1a) in MCF7 cells but not in MDA-MB-231 cells (Fig 5.9).

These data have shown that zoledronic acid has a differential effect on follistatin secretion dependent upon the ER status of the tumour cell line, with ER-ve cell lines decreasing follistatin secretion in response to zoledronic acid, an effect not seen in ER+ve cell lines. Both ER-ve and ER+ve cells take up zoledronic acid, as shown by the accumulation of unprenylated Rap1a, however, addition of GGOH reduced the levels of unprenylated Rap1a in MCF7 cells but not MDA-MB-231 cells, suggesting that uptake of the drug may be greater in the MDA-MB-231 cells.

5.5.3 Effect of activin and follistatin on proliferation of ER-ve and ER+ve cell lines.

In order to evaluate the effect of activin and follistatin on proliferation in both ER-ve and ER+ve cell lines, a time course and dose response MTS proliferation assay was performed. Three doses of activin (60pg/ml, 1800pg/ml and 6000pg/ml) and 3 time-points were evaluated (day 1, 3 and 5) to compare change in proliferation in control medium alone +/- exogenous activin. Due to the endogenous secretion of follistatin from MDA-MB-231 cells, medium +/- activin was replaced every 24 hours, to ensure the exogenous added activin would not be neutralized by endogenous secreted follistatin. Both the ER-ve cell line MDA-MB-231 and the ER+ve cell line MCF7 showed a decrease in proliferation compared to control medium alone with addition of activin. This fall also showed a significant dose-dependent effect with increased inhibition of proliferation with increasing doses of activin at day 1 and 3 in both cell lines (Fig 5.10). These results indicate that both an ER-ve and ER+ve cell lines are responsive to the growth inhibitory effect of exogenous activin *in vitro*, however, these effects are lost at day 5.

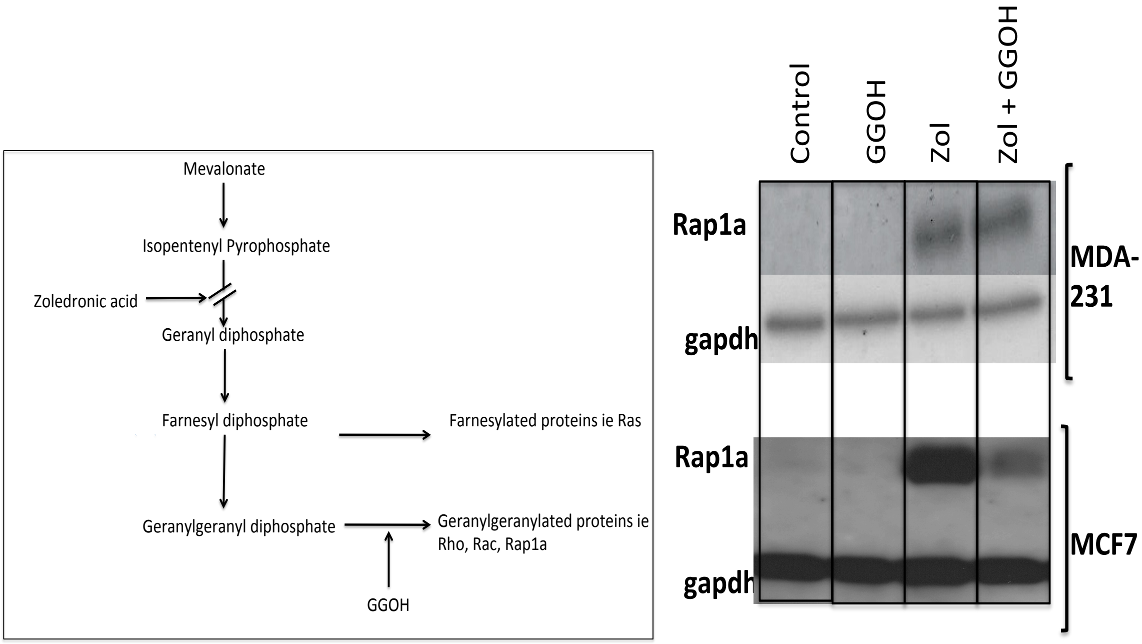


Figure 5.9. Effect of zoledronic acid on unprenylated Rap1a in MCF7 and MDA-MB-231 cells.

Cells were treated with medium alone, GGOH 50μM, ZOL 10μM or GGOH + ZOL for 48 hours. Rap1a primary antibody 1:200 was used to assess intracellular levels of unprenylated protein, with GAPDH 1:20,000 used as a loading control.

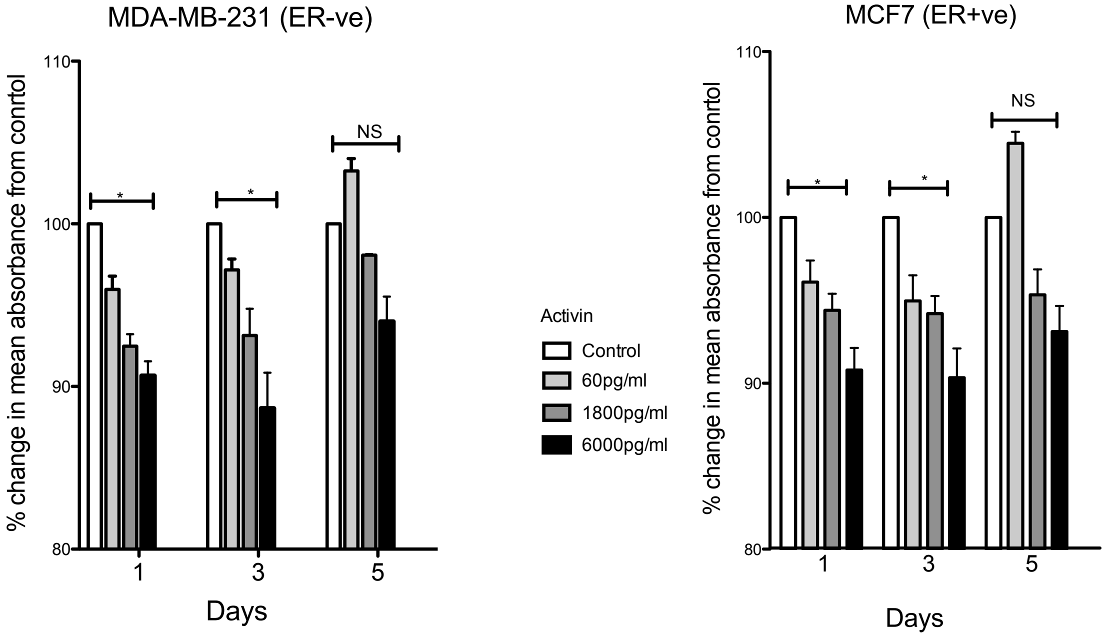


Figure 5.10. Effects of increasing doses of activin on tumour cell proliferation over time.

MDA-MB-231 and MCF7 cells were plate in 96 well plates and treated with increasing doses of activin over 5 days. Medium +/- activin was replaced every 24 hours with addition of MTS solution at the final time-points to allow measurement of absorbance of each dose of activin relative to untreated cells. Data represents 8 replicates and 3 repeats. Wilcoxon Signed-Rank test was used to detect differences between different doses, * p value <0.05, NS = no significant difference.

To confirmation the inhibition of cell growth was due to the exogenous activin, cell lines were treated with activin 6000pg/ml +/- an ALK4/5 inhibitor SB-431-542 (10μM/l) for 72 hours. The ALK4/5 inhibitor will prevent ActRII dimerising with its type I receptor ALK4. MDA-MB-231 cells showed less inhibition of proliferation with addition of the ALK4/5 inhibitor to activin, although this was not statistically significant, however, MCF7 cells showed increased inhibition of proliferation with addition of the ALK4/5 inhibitor to activin (Fig 5.11). The apparent increase in inhibition of proliferation in MCF7 cells was unexpected, and therefore direct toxicity of the ALK4/5 inhibitor on MCF7 cells was considered. Exposure of MCF7 cells to the ALK4/5 inhibitor for 3 days significantly decreased viable cell count compared to control (p value 0.005) (Fig 5.12). These data confirmed the growth inhibitory effects of activin in MDA-MB-231 cells.

To evaluate the effect of the exogenous activin neutralizer, follistatin, on the growth inhibitory effect of activin, cells were treated with activin +/- follistatin for 72 hours. The dose of follistatin that would be predicted to completely neutralize 6000pg/ml of activin is 64,000pg/ml. There was a reversal of the inhibitory effects of activin on cell proliferation when follistatin was added at a neutralizing dose in both MDA-MB-231 and MCF7 cells, although the changes were not statistically significant (Fig 5.13). These data indicated that both ER+ve and ER-ve cell lines are sensitive to the growth inhibitory effects of activin, which can be negated by follistatin.

5.5.4 Effect of Zoledronic acid on the downstream activin-signaling pathways.

Downstream activin-signaling pathways involve the receptor associated and cytoplasmic Smads 2 and 3, which translocate to the nucleus after phosphorylation to affect DNA transcription. The function of phosphorylated Smad2 depends upon its phosphorylation site, with COOH-tail phosphorylated Smad2 (pSmad2C) being a tumour suppressor and linker phosphorylated Smad2 (pSmad2L) promoting tumour growth (Matsuzaki 2011). Receptor binding of activin leads to phosphorylation of the membrane bound pSmad2C.

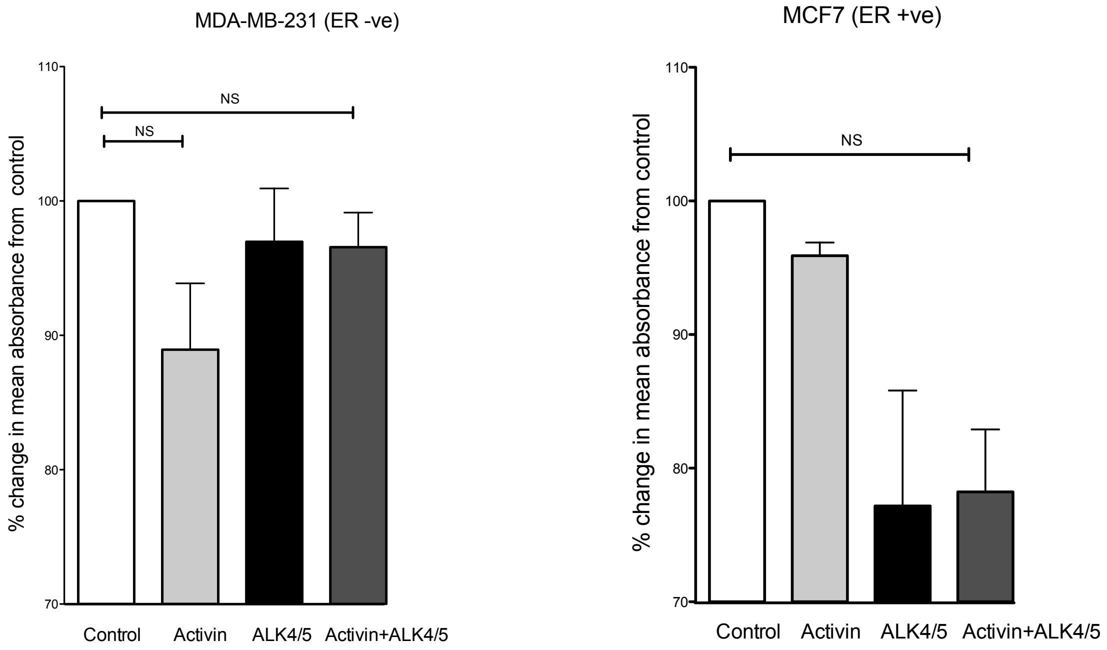


Figure 5.11. Effect of activin and the ALK4/5 inhibitor on cell proliferation at 72 hours.

Cells were plated in 96 well plates and treated with medium alone (control) or recombinant activin (6000pg/ml) +/-ALK4 inhibitor (10μM/l). The MTS solution was added at 72 hours. Data represents 8 replicates and 3 repeats. Wilcoxon Signed-Rank test for significance, NS= not significant.

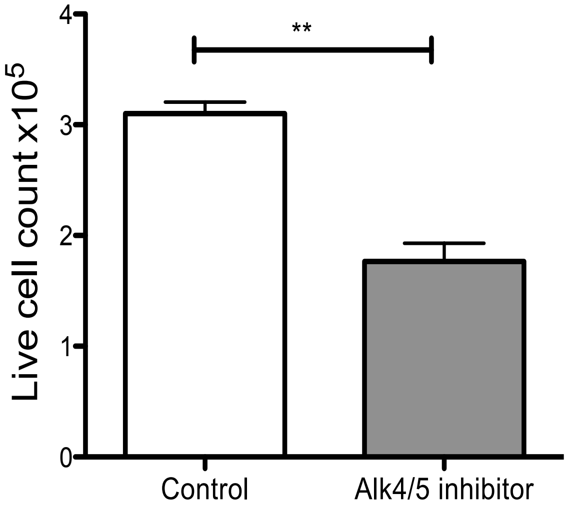


Figure 5.12 Effect of the ALK4/5 inhibitor on cell viability of MCF7 cells treated for 72 hours.

Cells were plated in 6 well plates and treated with medium alone (control) or medium + 10μM/l of ALK4/5 inhibitor. Data represents 3 replicates and 3 repeats. Mann Whitney test for significance ** p value <0.01.

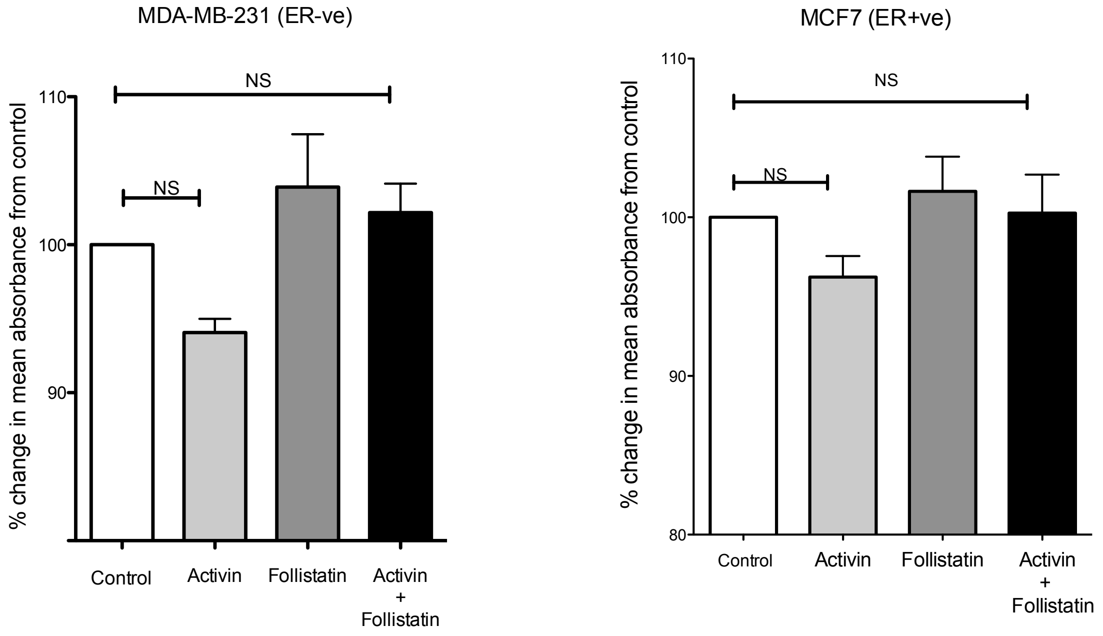


Figure 5.13. Effect of activin and follistatin on cell proliferation at 72 hours.

Cells were plated in 96 well plates and treated with medium alone (control) or medium with addition of exogenous recombinant activin (6000pg/ml) +/- follistatin (64,000pg/ml). The MTS solution was added after 72 hours. Data represents 8 replicates and 3 repeats. Wilcoxon Signed-Rank test for significance, NS= not significant.

In contrast, phosphorylation of pSmad2L occurs downstream of cytoplasmic proteins such as RAS and nuclear proteins such as cyclin dependent kinases (Matsuzaki 2011). Zoledronic acid, as an inhibitor of post-translational prenylation of small GTPases, can decrease the activity of RAS in breast tumour cells (Ibrahim, Mercatali *et al.* 2012), therefore may potentially decrease downstream phosphorylation of pSmad2L (Fig 5.14).

In order to evaluate if 50 μM zoledronic acid can modify phosphorylation pSmad2L in MDA-MB-231 and MCF7 cells, nuclear localization of the different phosphorylation sites was assessed using immunofluorescent staining (chapter 2 section 2.2.2.5) after 48 hours of treatment with zoledronic acid compared to medium only control. The percentage of MDA-MB-231 cells with nuclear pSmad2L was significantly decreased after 48 hours exposure to 50μM zoledronic acid compared to control (p value <0.0001). No significant difference was seen at the 24-hour time-point in MDA-MB-231 cells, or in the MCF7 cells at 24 or 48 hours (Fig 5.15 a and b).

To assess if the observed effect was an alteration in localization in response to zoledronic acid or a decrease in the total cellular quantity, western blots were performed on lysates from MDA-MB-231 cells treated with or without zoledronic acid for 48 hours. No significant alteration in the total cellular quantity of pSmad2L in response to zoledronic acid was seen (Fig 5.16), suggesting that zoledronic acid alters localization of pSmad2L but not total cellular quantity. No significant difference was seen in the percentage of cells with nuclear pSmad2C in either cell line at 48 hours (Fig 5.17 a and b). However, previous data had shown 48 hours of treatment with 50μM zoledronic acid decreased MDA-MB-231 secretion of the activin neutralizer, follistatin. Therefore to evaluate if the changes in follistatin secretion from MDA-MB-231 could affect the receptor associated phosphorylation of Smad2/3C, a cell based fluorescent immunoassay was used to evaluate the percentage of pSmad2/3C compared to total Smad2/3 in MDA-MB-231 cells exposed to 1 hour of supernatant from MDA-MB-231 cells that had been previously treated for 48 hours with 50μM zoledronic acid or medium alone. MDA-MB-231 cells exposed to supernatant from zoledronic acid treated cells had a significantly higher percentage of pSmad2C than cells treated for 48 hours with medium alone (p value 0.0286).

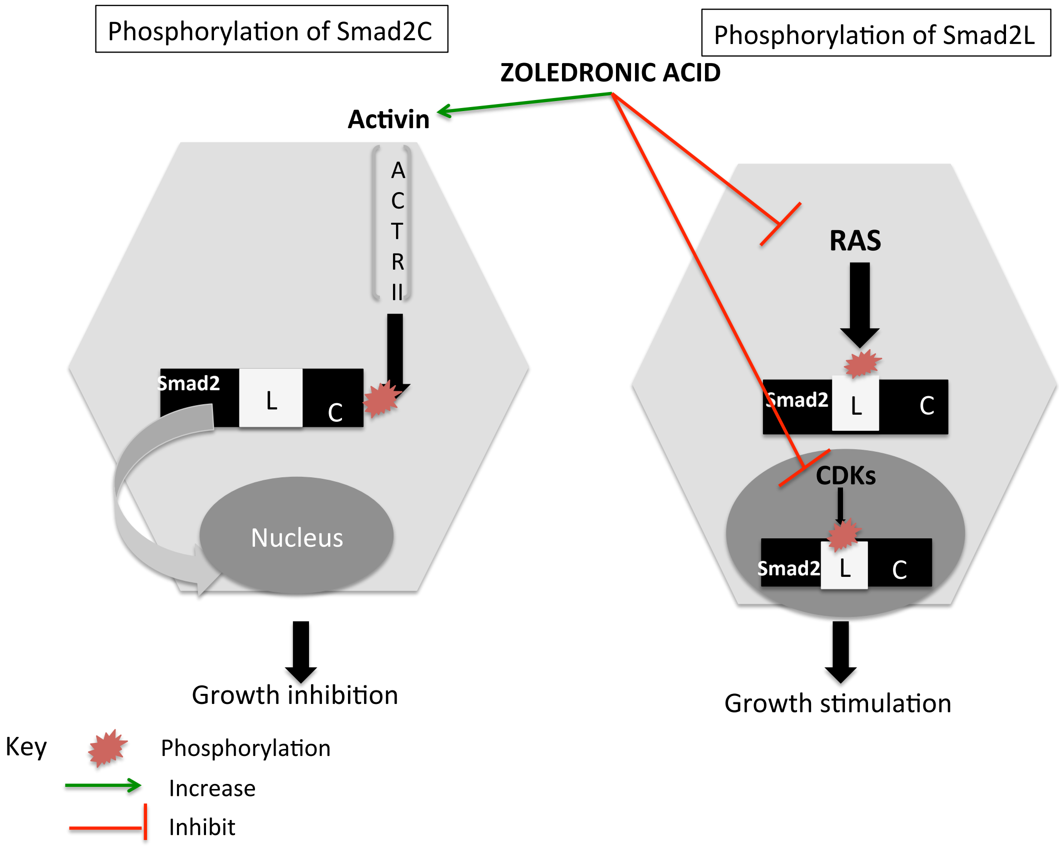


Figure 5.14. Schematic of the different effector functions of the alternate phosphorylation sites of Smad2 in tumour cells, and the potential effect of Zoledronic acid.

COOH-tail phosphorylation of Smad2 (C) results from dimerization of the ACRII receptor with ALK4, allowing translocation to the nucleus to inhibit tumour cell growth. Phosphorylation at the linker region (L) changes the effector function of Smad2, which becomes a tumour promoter. ZOL increases the bioavailability of activin in ER-ve cell lines by decreasing the activin neutralizing molecule, follistatin. ZOL may also be able to affect linker phosphorylation via its known effects on small GTPases such as RAS and the cyclin dependent kinases (CDKs).

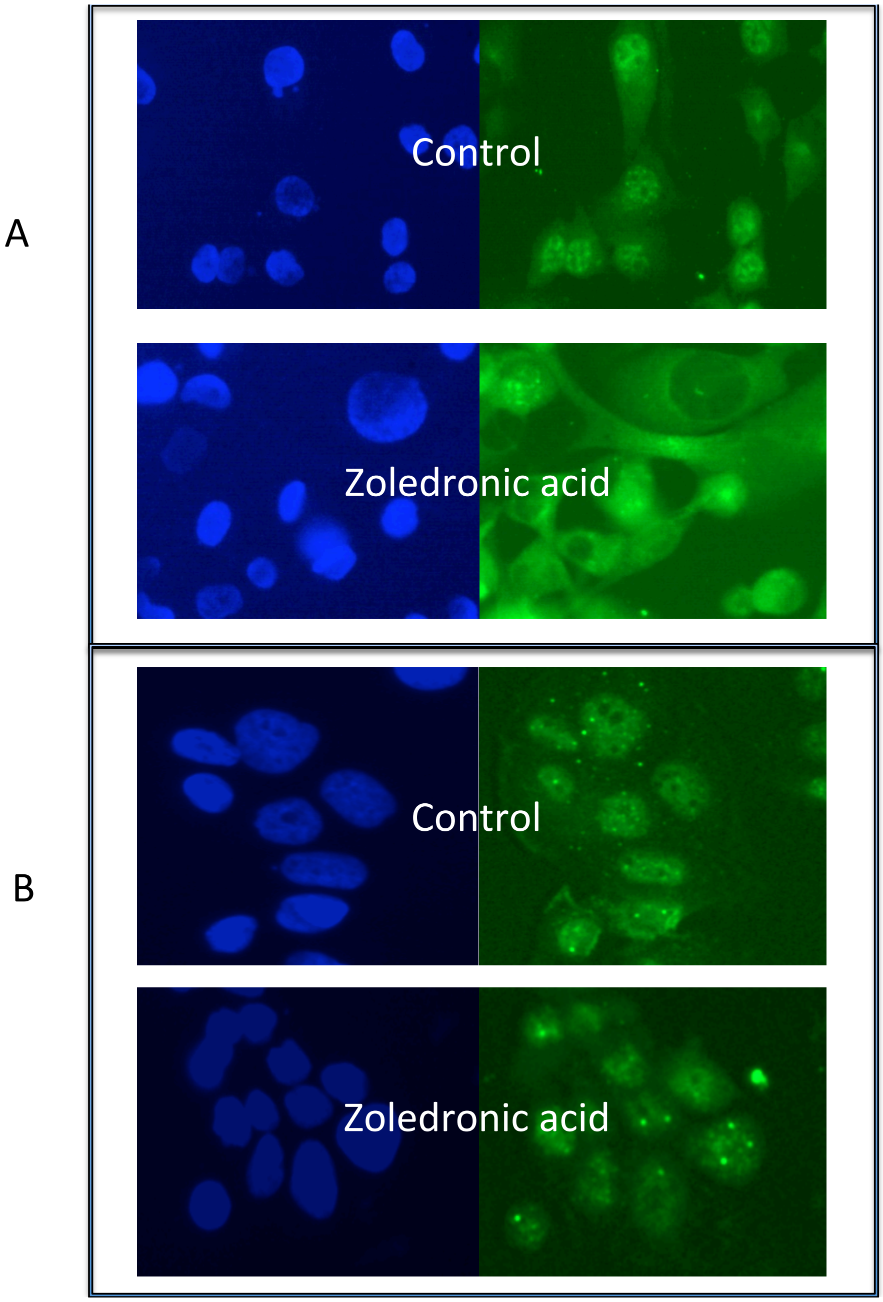


Figure 5.15a. Representative immunofluorescent images of the effect of zoledronic acid on cellular localization of pSmad2L in MDA-MB-231 and MCF7 cells.

Corresponding Dapi (blue) and pSmad2L (green) images in (**A**) MDA-MB-231 cells treated with medium alone (control) and 50μM zoledronic acid for 48 hours. (**B**) MCF7 cells treated with medium alone and 50μM zoledronic acid for 48 hours.

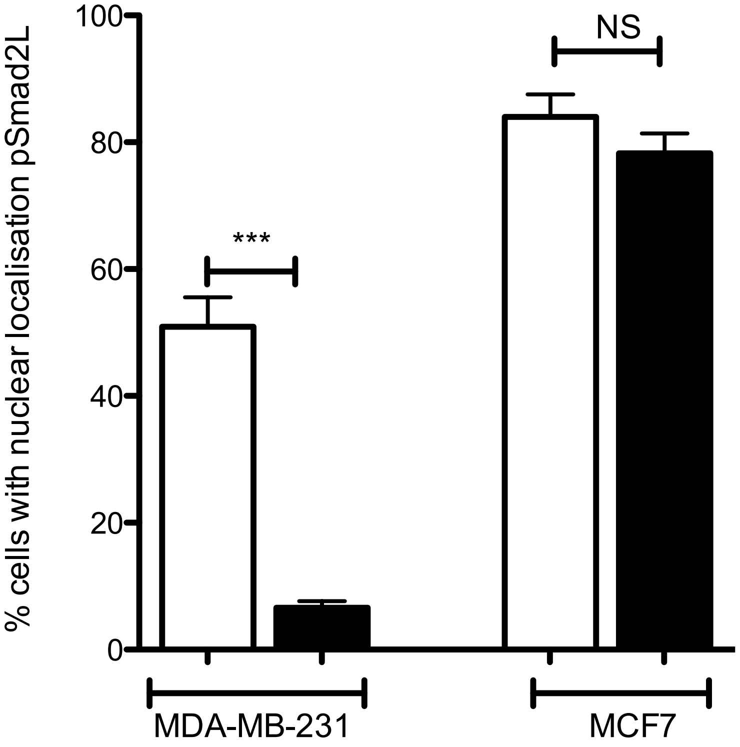
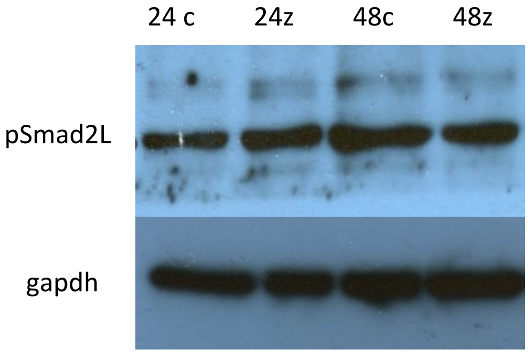


Figure 5.15b. Effect of zoledronic acid on nuclear localization of pSmad2L in MDA-MB-231 cells and MCF7 cells.

A minimum of 100 cells per chamber were counted from MDA-MB-231 and MCF7 treated with medium alone (open bars) or medium + 50μM zoledronic acid (closed bars) for 48 hours. Data represents mean + SEM of 3 replicates and 3 repeats. Mann Whitney test for significance ***p value <0.001, NS=not significant



Key; 24 = 24 hours
48 = 48 hours
c= control medium alone
z= medium + zoledronic acid 50µM

Figure 5.16. Effect of zoledronic acid on the total cellular quantity of pSmad2L in MDA-MB-231

Cells were plated in 6 well plates and treated with medium alone (c) or medium + 50µM zoledronic acid (z) for 24 or 48 hours. Cells were lysed at each time-point and processed to western blot using specific antibodies to Smad2L 1:1000 and gapdh 1:20,000 as a loading control.

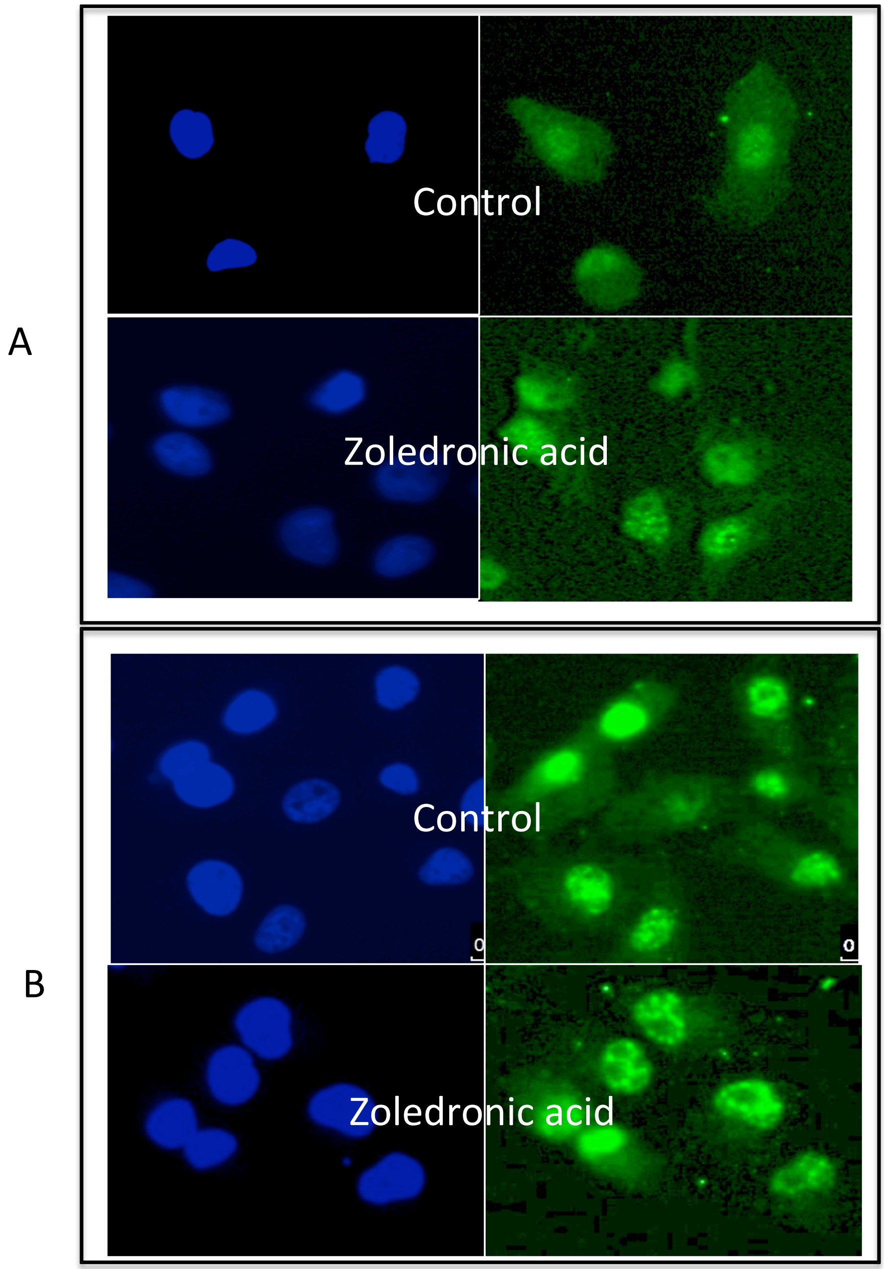


Figure 5.17a. Representative immunofluorescent images of the effect of zoledronic acid on cellular localization of pSmad2C in MDA-MB-231 and MCF7 cells.

Corresponding Dapi (blue) and pSmad2C (green) images in (**A**) MDA-MB-231 cells treated with medium alone (control) and 50μM zoledronic acid for 48 hours. (**B**) MCF7 cells treated with medium alone and 50μM zoledronic acid for 48 hours.

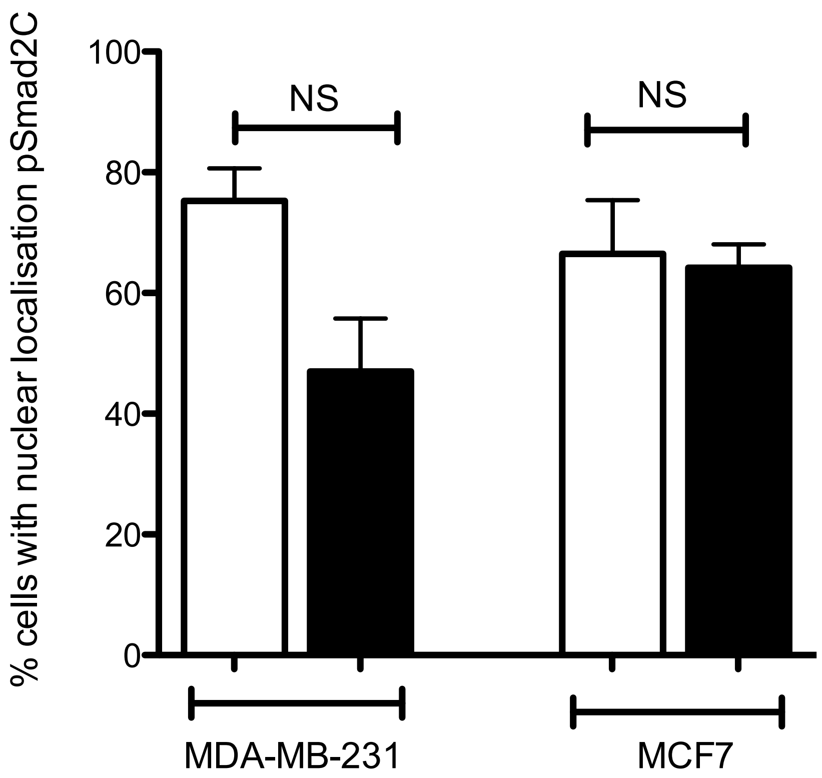


Figure 5.17b. Effect of zoledronic acid on nuclear localization of pSmad2C in MDA-MB-231 cells and MCF7 cells.

A minimum of 100 cells per chamber were counted from MDA-MB-231 and MCF7 cells treated with medium alone (open bars) or medium+50μM zoledronic acid (closed bars). Data represents mean + SEM of 3 replicated and 3 repeats. Mann Whitney test for significance NS = not significant.

Since the supernatant would have contained zoledronic acid, a further control was added of 1-hour treatment of 50μM zoledronic acid which did not show any change in the percentage of pSmad2C compared to medium alone (Fig 5.18). Phosphorylation of Smad2C is not solely the result of activin binding to ActRII but can also be increased by TGFβ1 binding to TGFβRII. To assess if a 48-hour treatment with 50μM zoledronic acid altered TGFβ1 secretion in MDA-MB-231 cells, TGFβ1 levels from the cell supernatant were evaluated and compared to control medium alone using a TGFβ1 ELISA. Zoledronic acid did not significantly alter the secretion of TGFβ1 from MDA-MB-231 cells at 48 hours (Fig 5.19), therefore the effect of the supernatant from zoledronic acid treated MDA-MB-231 cells on pSmad2C, is likely to be due to a decrease in follistatin levels which increases bioavailability of activin.

These data therefore suggest that zoledronic acid can both decrease nuclear localization of pSmad2L and increase cellular quantity of pSmad2C in MDA-MB-231 cells, supporting the potential increased anti-tumour effects in ER-ve breast cancer cells.

5.5.5 Effect of zoledronic acid on follistatin and linker phosphorylated Smad2 in a sub-cutaneous xenograft model of ER-ve MDA-MB-436 tumours.

To evaluate if the zoledronic acid induced changes in both follistatin levels and pSmad2L in ER-ve cells *in vitro* also occurred *in vivo*, ER-ve MDA-MB-436 sub-cutaneous tumour sections from mice treated with or without zoledronic acid were stained and scored for follistatin and pSmad2L.

Both MDA-MB-231 and MDA-MB-436 sub-cutaneous tumours express follistatin and pSmad2L (Fig 5.20a and b). To assess the effects of zoledronic acid on follistatin, MDA-MB-436 tumour sections from saline (n=5) and zoledronic acid (n=6) treated mice were scored for intensity of staining and area of positive staining. Follistatin staining was primarily cytoplasmic in the tumor sections, with the intensity of stain relating to the distance from the centre of the tumour. Large tumours with central necrotic areas demonstrated no staining in the core, whereas the outer cortex of the tumours demonstrated more intense staining in both control and zoledronic acid treated tumours (Fig 5.21a). No difference was seen in the intensity of follistatin stain, however, there was

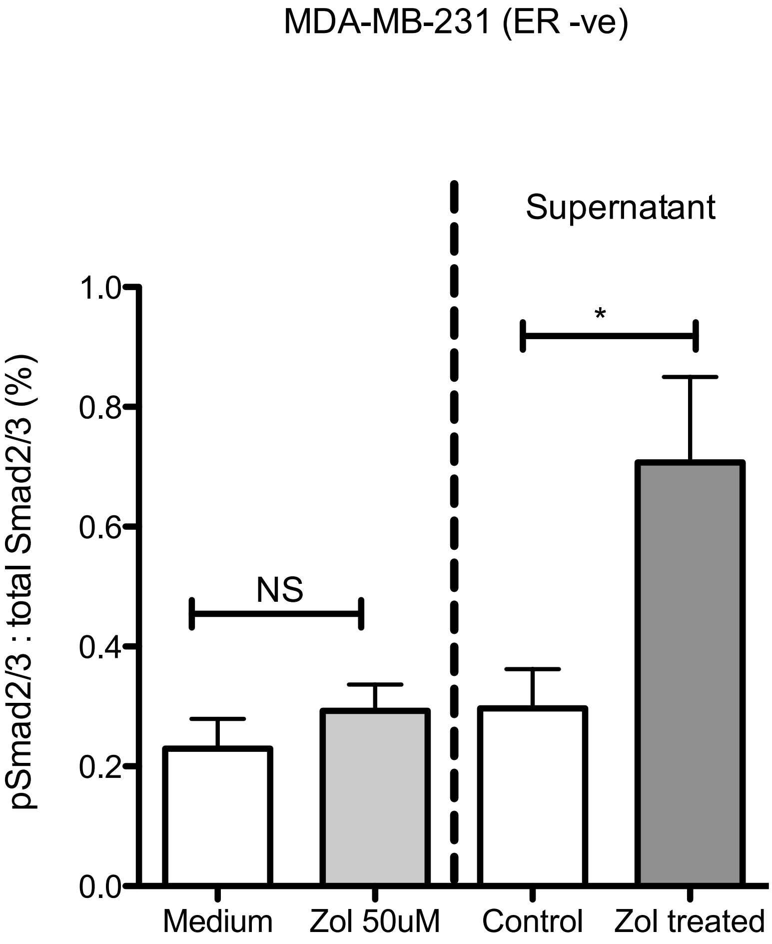


Figure 5.18. Effect of supernatant from zoledronic acid treated MDA-MB-231 cells on intracellular pSmad2/3 levels in MDA-MB-231 cells.

MDA-MB-231 cells were plated in a 96 well plate and exposed to either supernatant from MDA-MB-231 cells previously treated with 50μM zoledronic acid for 48 hours, or fresh medium + 50μM zoledronic acid for 1 hour. Cells were fixed and fluorescent antibodies used to detect pSmad2/3C and total Smad2/3C. Data represent the % of pSmad2C to total Smad2C +SEM, and represents 3 replicates from 3 repeated cell secretion experiments. Mann Whitney test for significance, * p value <0.05, NS= not significant.

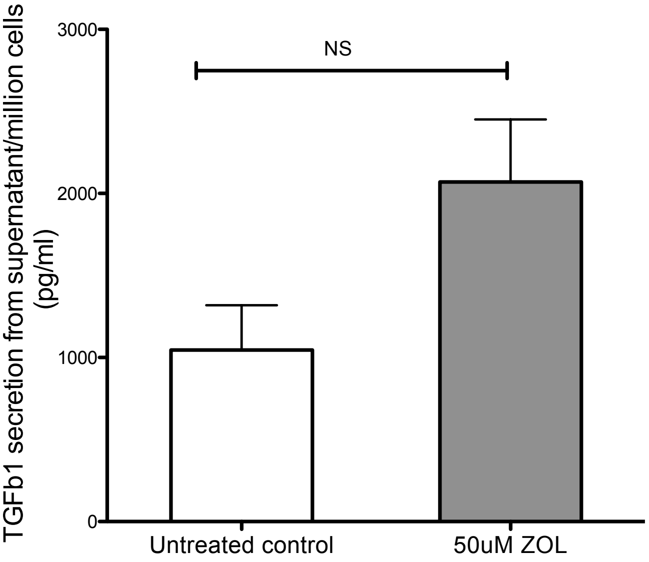


Figure 5.19 Secretion of TGFβ1 from MDA-MB-231 cells in response to zoledronic acid.

MDA-MB-231 cells were plated in 6 well plates and treated with medium alone (control) or medium + 50μM zoledronic acid for 48 hours. Supernatant was removed and processed to TGFβ1 ELISA. Data represents mean +SEM of 3 replicates and 3 repeats. Mann Whitney test for significance, NS=not significant.

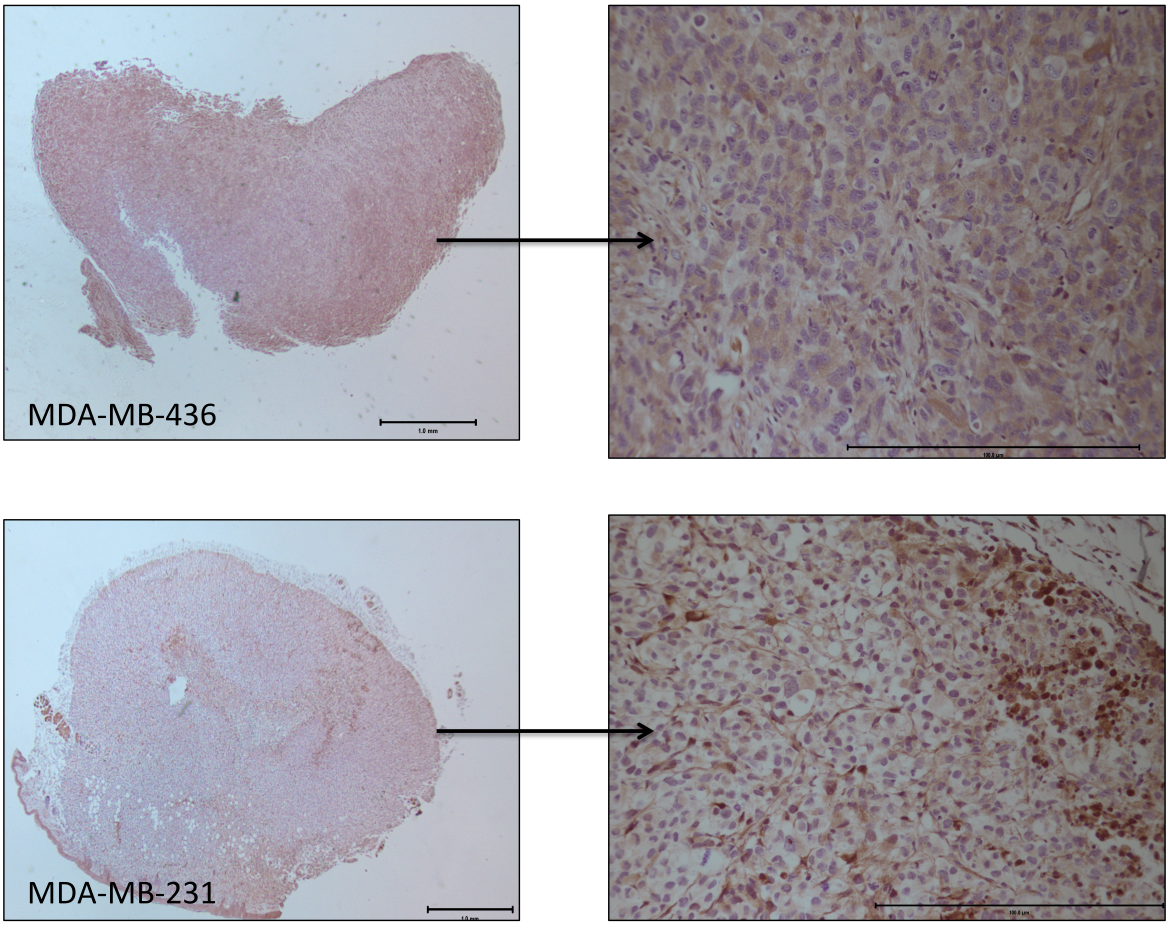


Figure 5.20a Representative images showing follistatin expression in MDA-MB-231 and MDA-MB-436 sub-cutaneous tumours

The positive follistatin staining was located in the cytoplasm and extracellular space in both ER-ve tumours. Images represent x1.6 magnification (left) and x20 magnification (right).

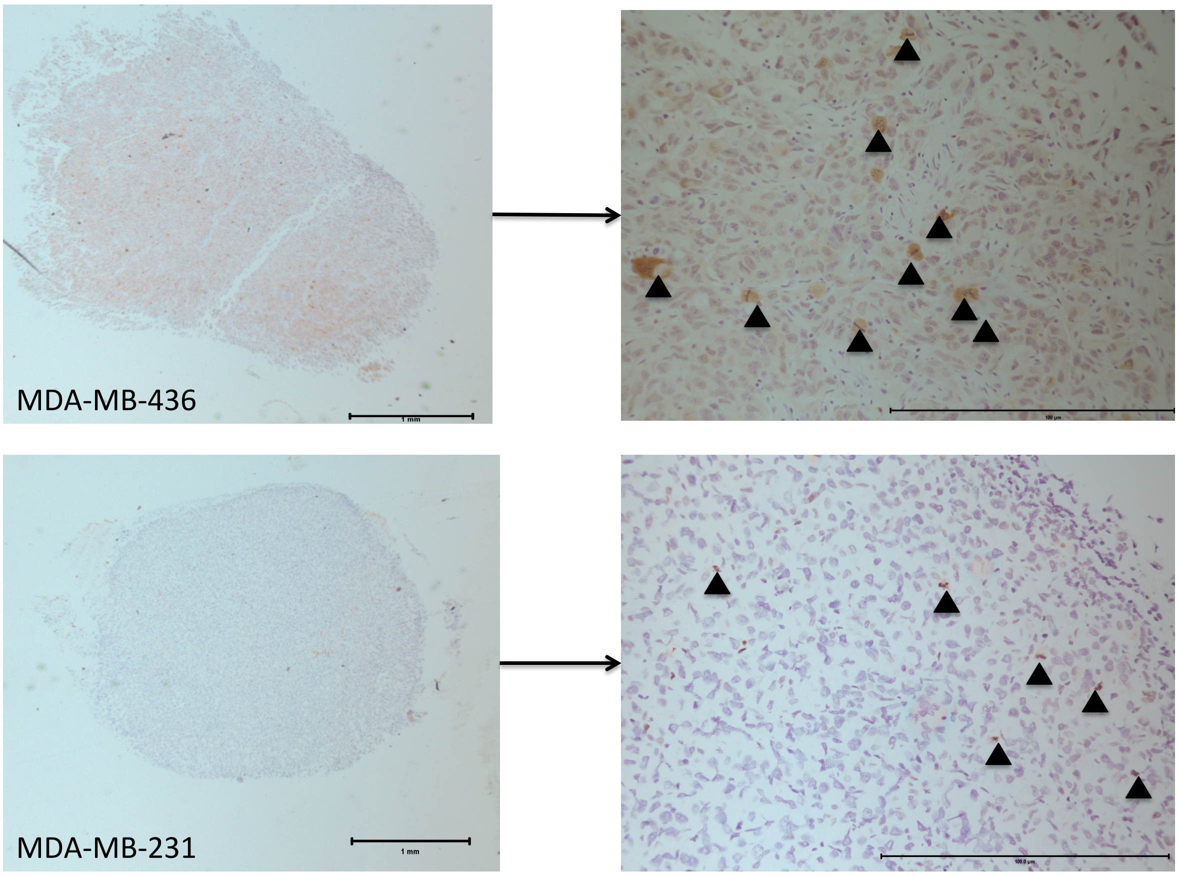


Figure 5.20b Representative images showing pSmad2L expression in MDA-MB-231 and MDA-MB-436 sub-cutaneous tumours

The positive pSmad2L staining was primarily located in mitotic cells in both ER-ve tumours (highlighted by black triangles). Images represent x1.6 magnification (left) and x20 magnification (right).

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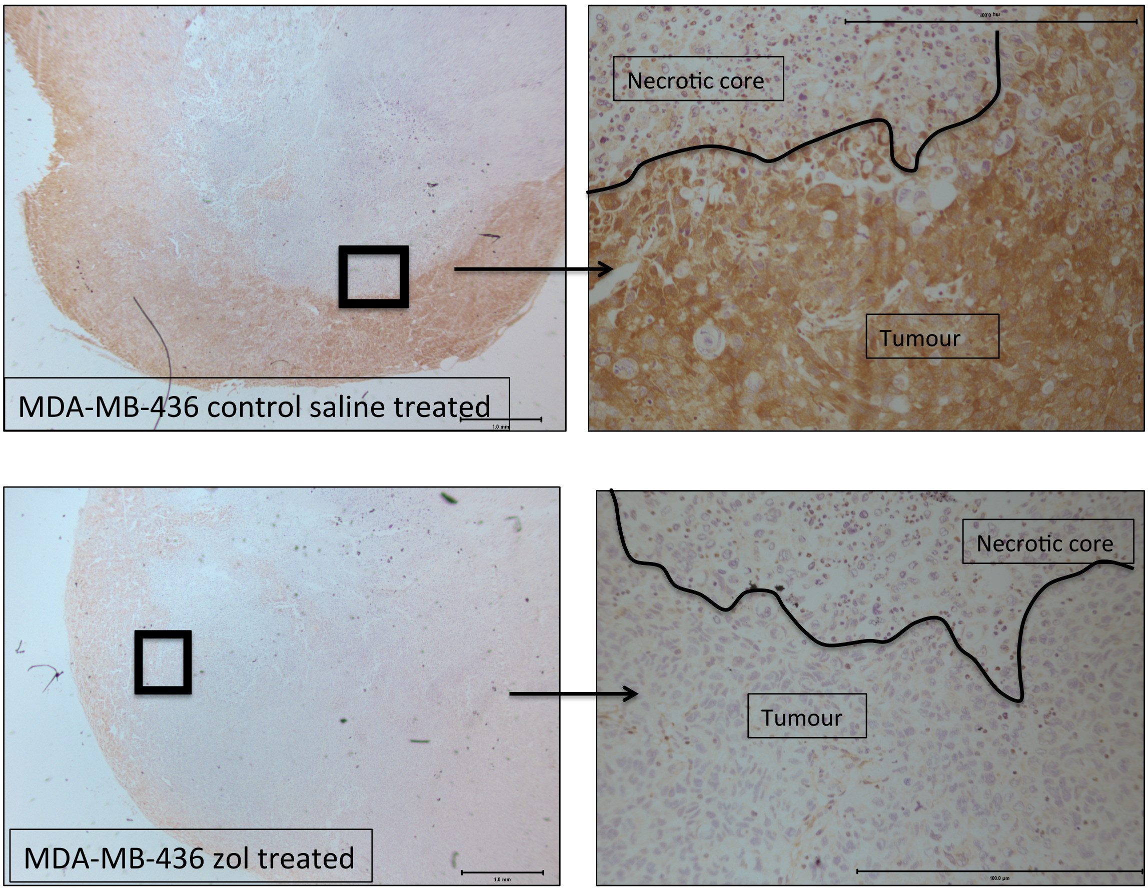


Figure 5.21a Representative images of follistatin expression in MDA-MB-436 sub-cutaneous tumours from mice treated with and without zoledronic acid.

The positive follistatin staining was localized to the outer cortex of the tumours with no positive staining detected in the necrotic core. For quantification of staining, 20x750μm2 images were scored from two sections per tumour and scored for intensity of +ve stain and area of positive stain on a 0-3 scale. Images represent tumours from saline treated mice (top) and zoledronic acid treated mice (bottom) at x1.6 magnification (right) and x20 magnification (left).

a significant decrease in the area of staining in tumours from mice treated with zoledronic acid compared to saline (Fig 5.21b). Data was analysed using average scores from 2 assessors blinded to treatment groups.

For pSmad2L, MDA-MB-436 tumour sections were assessed from saline (n=6) and zoledronic acid treated mice (n=8). The results showed that when there was +ve staining for pSmad2L, the cells were in mitosis (Fig 5.22a). The number of cells with positive staining for pSmad2 was significantly lower in zoledronic acid treated mice (Fig 5.22b). These tumours had previously been stained and scored for the proliferation marker Ki67, with no significant difference in Ki67 seen between saline and zoledronic acid treated mice (Ottewell, Monkkonen *et al*. 2008). Also further work from the same group evaluating the effects of a similar 6-week schedule of zoledronic acid on sub-cutaneous MDA-MB-436 tumours using cell cycle specific microarrays, had shown no significant change in genes encoding for cyclins and CDKs with single agent zoledronic acid (Ottewell, Lefley *et al*. 2010). Therefore the number of cells in mitosis would not be expected to be different between tumours from the control and zoledronic acid treatment groups, indicating the decrease in pSmad2L is not secondary to alterations in mitosis levels.

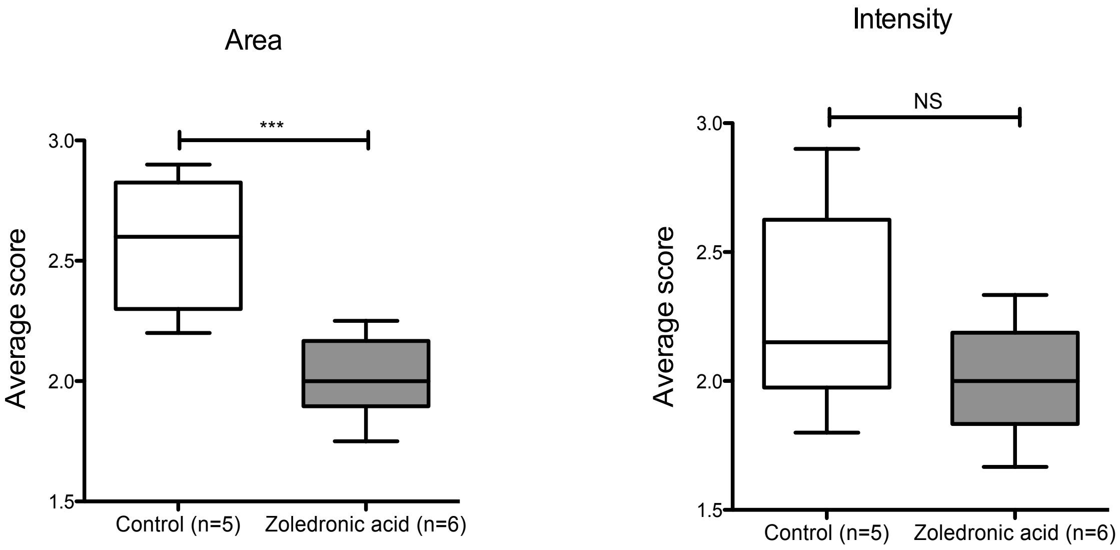


Figure 5.21b Effect of zoledronic acid on follistatin expression in sub-cutaneous MDA-MB-436 tumours.

Area of +ve staining was scored as 0 no stain, 1 up to 10% +ve cells, 2 10-50% +ve cells and 3 >50% +ve cells.
Intensity of staining was scored as 0 no stain, 1 weak, 2 moderate and 3 strong.
20 different tumour areas were evaluated per animal and data represents average score from 2 persons blinded to treatment. Mann Whitney test for significance *** p value <0.001, NS= not significant.

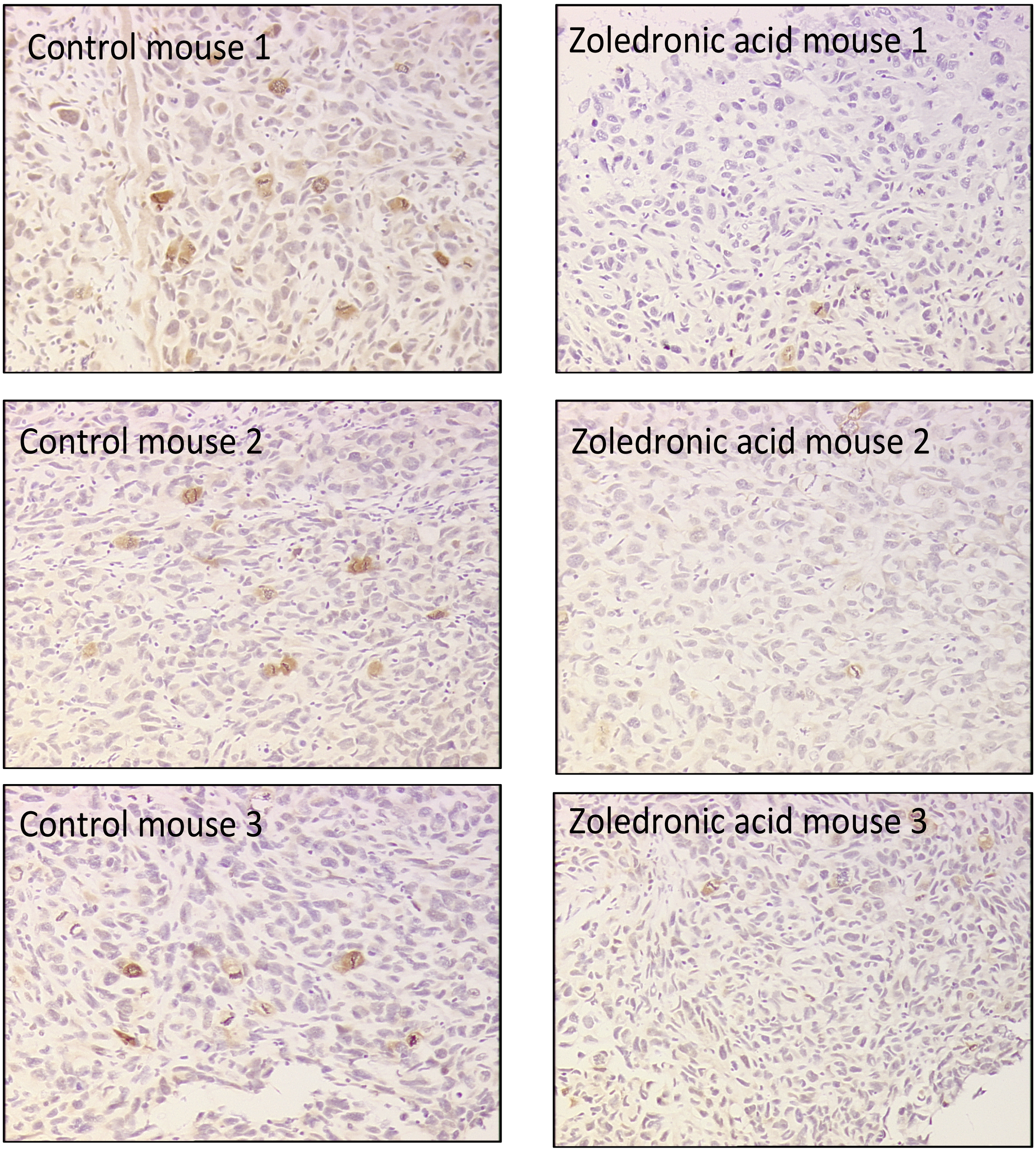


Figure 5.22a. Representative images of pSmad2L expression in MDA-MB-436 sub-cutaneous tumours in mice treated +/- zoledronic acid

Sections from sub-cutaneous tumours were stained for follistatin with primary antibody 1:200. Representative examples from control (saline treated) (left) vs. zoledronic acid treated mice (right).

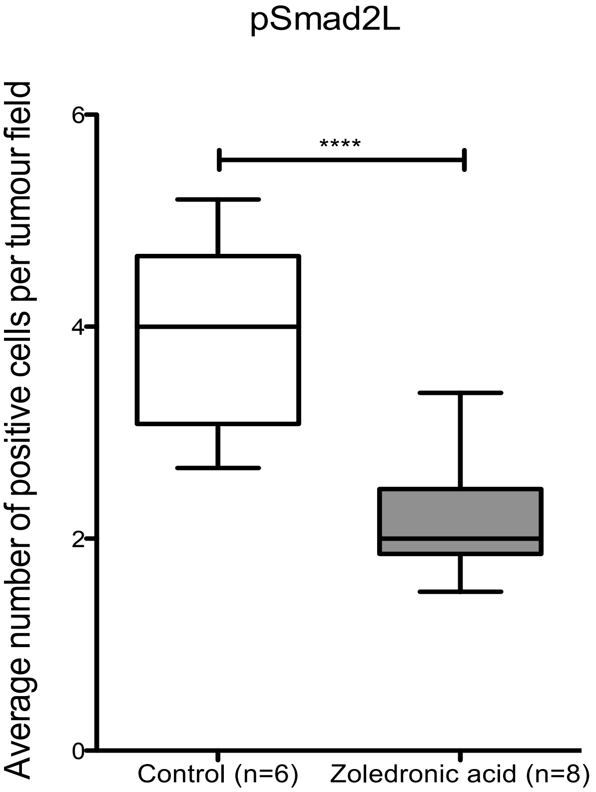


Figure 5.22b. Effect of zoledronic acid on pSmad2L expression in MDA-MB-436 sub-cutaneous tumours

20 images were taken from different areas of each tumour. The number of positive cells was counted per area and averaged per tumour. **A** Data in graph represents average score per treatment group in control (saline treated) vs. zoledronic acid treated. Mann Whitney test for significance *** p value <0.001

5.6 Discussion

The results presented in this chapter support a potential new novel anti-tumour mechanism of action of zoledronic acid in ER-ve breast cancer cells, via effects on the activin-signaling pathway. Zoledronic acid caused an apparent increase in the bioavailability of activin by decreasing the activin neutraliser, follistatin, both *in vitro* and *in vivo*. Zoledronic acid also appeared to reduced the nuclear localization *in vitro,* and the number of positive cells *in vivo,* of the tumour promoter pSmad2L. These data may represent a mechanism to explain the differential anti-tumour effect of zoledronic acid according to ER receptor status, which has been demonstrated in neo-adjuvant clinical trials of zoledronic acid.

Both activin and follistatin were secreted from ER-ve and ER+ve breast cancer cells, but in different molar ratios, with ER-ve cells secreting an excess of follistatin:activin favouring tumour proliferation, compared to ER+ve cells which secreted an excess of activin:follistatin. Previously published data have demonstrated that activin is secreted by benign proliferative fibrocystic disease of the breast (Di Loreto, Reis *et al*. 1999). The activin βA subunit is detected in breast carcinoma, fibroadenoma and normal mammary tissue with a 43% increase in mRNA levels in carcinoma compared to normal breast tissue, translating into a significantly higher tissue concentration of activin A in breast cancer homogenates compared to non-neoplastic breast tissue (Reis, Cobellis *et al*. 2002). Neither of these studies evaluated any concurrent expression of follistatin mRNA or follistatin secretion from normal mammary or malignant tissue.

Whilst activin is generally accepted to be a tumour suppressor in breast carcinoma, and confirmed by my data, there is controversy in the literature regarding the ability of activin to affect ER-ve cell lines, with some reports of insensitivity of ER-ve cell lines to the growth inhibitory effects of activin. Kalkhoven *et al* evaluated a panel of breast cancer cell lines for their growth sensitivity to exogenous TGFβ and activin A and showed that growth of the ER+ve cell lines MCF7, T47D and ZR75-1 was inhibited by activin, whereas ER-ve cell lines including MDA-MB-231, MDA-MB-436 were resistant to activin growth inhibition. The reason for the lack of growth inhibition in MDA-MB-231 and MDA-MB-436 cell lines was not due to lack of activin receptors, or ability of activin to bind to receptors, and the authors suggested the resistance to the anti-proliferative effects of activin may be located downstream of the activin receptor (Kalkhoven, Roelen *et al*. 1995). However, the presence and/or effect of an exogenous secreted activin neutralizer such as follistatin was not measured in this study. Burdette *et al* evaluated the effects of activin, follistatin and an ALK4/5 inhibitor in the ER+ve cell lines MCF7 and T47D. Proliferation was significantly inhibited by activin, which was negated with addition of an ALK4/5 inhibitor. Activin also increased the number of T47D cells which were in G0-1 phase, and the addition of follistatin reduced the number of G0-1 cells by 30% with a concurrent increase in the number of cells in S phase (Burdette, Jeruss *et al*. 2005), confirming the ability of follistatin to negate the tumour suppressor action of activin.

Jeruss *et al* compared components of the activin signaling pathway in grade 1 (n=22), grade 2 (n=18) and grade 3 (n=19) breast cancer, evaluating activin ligand subunits (βA and βB), receptors (ActRIIA, ActRIIB and ActRIB) and downstream signaling proteins (Smad 2,3,4). Using immunohistochemistry, activin βA subunit was detectable in all tumour grades and in normal breast tissue, and there was no difference in staining between the benign and malignant cells. Both activin type II receptors were attenuated in grade 3 tumours compared to grade 1 and 2, and using immunofluorescent imaging, nuclear localization of all Smads were lower in grade 3 tumours compared to grade 1 and 2. These data indicate that as tumour grade increases the cellular production of activin A remains constant, however, the receptor and downstream signaling pathways are diminished. This confirmed the tumour suppressive role of activin in breast cancer (Jeruss, Sturgis *et al*. 2003). Interestingly, in this study ER+ve tumours had a significantly higher nuclear Smad3 staining compared to ER-ve tumours (77% vs 13% p=<0.001), but again follistatin was not measured. From my data it seems plausible that a common mechanism of resistance to activins tumour suppressor actions in ER-ve breast cancer, may be linked to breast cancer cell secretion of activin neutralizing molecules such as follistatin.

Activin's bioavailability is regulated by several proteins, including follistatin and follistatin related gene (FLRG). Razanajoana *et al* showed that FLRG is expressed by breast cancer cells, and follistatin expression was detected in the two ER-ve cell lines MDA-MB-459 and HS578T cells. The anti-proliferative effect of activin was weak in ER+ve MCF7 cells and undetectable in ER-ve MDA-MB-436 cells cultured over 4 days.

However, after washing cells to remove endogenous secreted inhibitors such as follistatin and FLRG, Smad2C was phosphorylated in response to activin in all cell lines. After silencing FLRG expression in MCF7 and MDA-MB-436 cells, expression of pSmad2C was increased, and the proliferation of both cell lines was decreased. These results suggest that in both ER-ve and ER+ve cell lines, endogenous activin inhibitors such as FLRG can promote cell growth by neutralizing the inhibitory effects of activin (Razanajaona, Joguet *et al*. 2007). Using immunohistochemistry to detect follistatin and FLRG in clinical samples of breast proliferative disorders, follistatin expression was significantly increased in the stromal cells of fibroadenomas compared to normal breast tissue, and FLRG staining was significantly higher in invasive carcinoma compared to normal breast tissue (Bloise, Couto *et al*. 2009). These data suggest that tumour cells may use activin inhibitors such as follistatin and FLRG to reduce or even negate the anti-proliferative effects of activin.

The differential effects of zoledronic acid on follistatin secretion according to ER status of breast cancer cell lines, has not been previously reported. However, other studies have shown a differential effect of zoledronic acid on proliferation of ER-ve and ER+ve cells via alternative mechanisms. Zoledronic acid used in combination with cisplatin had an enhanced anti-proliferative effect in ER-ve cell lines but not the ER+ve cells via alteration in p21, pMAPK and m-TOR pathways (Ibrahim, Liverani *et al*. 2013). Rachner *et al* confirmed the differential effect of zoledronic acid on proliferation according to ER status of cell lines showing that MCF7 cells did not demonstrate a significant change in proliferation in response to increasing doses of zoledronic acid whereas MDA-MB-231 cells showed a dose- dependent inhibition of proliferation and increase in apoptosis via activation of caspase 3 and 7 (Rachner, Singh *et al*. 2010). These results were supported by Fehm *et al* who showed the antitumour effect of zoledronic acid on primary breast cancer samples removed at surgery (n=116), was greatest in ER-ve tumours using an ATP-tumour chemosensitivity assay (Fehm, Zwirner *et al*. 2012). Some studies have reported ER+ve cells to be more responsive to zoledronic acid (Fromigue, Lagneaux *et al*. 2000), or no differential effect of zoledronic acid according to ER status (Senaratne, Pirianov *et al*. 2000; Jagdev, Coleman *et al*. 2001).

A possible explanation for the differential effects of zoledronic acid on proliferation according to ER status may be due to differences in uptake of the drug. Previously published data have shown that the maximal anti-tumour effects of zoledronic acid are obtained *in vivo* when unprenylated Rap1a is detected in tumour lysates (Ottewell, Woodward *et al*. 2009; Ottewell, Lefley *et al*. 2010). I detected unprenylated Rap1a in both MDA-MB-231 and MCF7 cells after treatment with the same dose of zoledronic acid, However, addition of the mevalonate intermediate GGOH partly reversed the accumulation of Rap1a in MCF7 cells only, suggesting the ER-ve MDA-MB-231 cells either take up more zoledronic acid, or do not take up the GGOH as effectively as MCF7 cells.

A decrease in follistatin secretion from breast cancer cells would be expected to increase activin binding to its receptor by increasing bioavailability of 'active' activin. This should then increase phosphorylation of the intracellular receptor associated pSmad2C protein. My data showed that supernatant from MDA-MB-231 cells, which had reduced follistatin levels, increased the percentage of pSmad2/3C relative to total Smad2/3. Phosphorylation of Smad2 via the canonical pathway, resulting in c terminus phosphorylation, has been shown to suppress breast cancer cell invasion and metastases to bone *in vitro* and *in vivo*. Knockdown of pSmad2C in MDA-MB-231 cells *in vitro* resulted in a lower growth rate and reduced migration compared to parental MDA-MB-231 cells. However, in a mouse model of bone metastases the Smad2 knockdown cells showed significantly faster establishment in bone compared to control, suggesting a tumour suppressive role for Smad2C *in vivo* (Petersen, Pardali *et al*. 2010).

These data are in contrast to a study which showed inhibition of phosphorylation of pSmad2C signaling using an AKl5 inhibitor, reduced metastases to lung from 4T1 (ER+ve) breast tumours (Park, Son *et al*. 2011), suggesting that inhibition of pSmad2C may have a differing anti-metastatic effect according to the site of metastases or breast cancer cell line used.

In clinical studies, a large tissue microarray study of breast tumours from 426 patients showed loss of pSmad2 was associated with a shorter overall survival (median survival 110.5 vs 306.5 weeks p=0.024), but did not correlate with tumour type, grade, receptor status or lymph node status, suggesting loss of pSmad2C may be an independent prognostic factor (Xie, Mertens *et al*. 2002). Similar results were shown in a study of 61 patients with T1-2 N0 tumours, confirming loss of pSmad2/3C was significantly associated with a shorter disease free survival (Koumoundourou, Kassimatis *et al*. 2007).

Phosphorylation of Smad2 at the linker region has been reported to alter the tumour suppressive action of Smad2 to a tumour promoter action. Phosphorylation at this site is primarily via cytoplasmic RAS and nuclear cyclin dependent kinases (Matsuzaki 2011), as opposed to the canonical receptor mediated activin pathway leading to COOH-terminus phosphorylation. I have shown that zoledronic acid can decrease linker phosphorylation of Smad2 in ER-ve breast cancer cells both *in vitro* and *in vivo*. Zoledronic acid has recently been shown to decrease RAS expression and activity in 2 ER-ve cell lines (MDA-MB-231 and BRC-230), with an associated inhibition of cell proliferation. ER+ve cell lines showed less effect of zoledronic acid on RAS expression and activity with no associated effect on cell proliferation (Ibrahim, Mercatali *et al*. 2012). Therefore a reduction is RAS activity could be expected to reduce RAS dependent linker phosphorylation of Smad2, as shown in my data.

These data have demonstrated a potential dual mechanism of action of zoledronic acid on the activin signaling pathway in ER-ve breast cancer cells; firstly via a decrease in follistatin secretion *in vitro* and *in vivo* leading to an increase in pSmad2C, and secondly via a decrease in phosphorylation of pSmad2L *in vitro* and *in vivo.* These data provide a possible further anti-tumour mechanism of action of zoledronic acid, that could contribute to the differential effect seen in clinical trials of ER –ve and +ve breast cancer.

Chapter 6. The ovarian hormone inhibin A, and zoledronic acid, influence levels of activin and follistatin in the tumour and bone microenvironment.

6.1 Summary

Inhibin A is an ovarian secreted hormone, which has been previously shown *in vitro* and *in vivo* to affect the bone microenvironment by increasing osteoblast activity and bone density. Supplies of the recombinant inhibin A protein extensively used in endocrine and bone research ceased to be commercially available several years ago, with no current gold standard recombinant protein used for *in vitro* or *in vivo* studies. Therefore I sought to establish the effect of a human recombinant inhibin A protein not previously reported in the literature, on breast cancer cells *in vitro* and on the bone microenvironment *in vivo.*

The effect of inhibin A and zoledronic acid on bone activin and follistatin levels has not been reported. The ratio of activin:follistatin may influence tumour cell proliferation, since follistatin can negate the anti-proliferative effects of activin in breast cancer cells. Levels of these paracrine factors in the bone microenvironment may have the potential to affect disseminated tumour cells that seed to bone in early breast cancer. The aim of the work described in this chapter was to evaluate the activity of a human recombinant inhibin A protein not previously used *in vivo* to assess effects on the bone microenvironment, and compare these to the effects of zoledronic acid in a murine bone metastases model. The direct effects of inhibin A on activin and follistatin secretion from breast cancer cells was also evaluated *in vitro*.

The results indicate that recombinant human inhibin A sourced from the National Institute for Biological Standards and Controls (NIBSC) is active *in vivo*, as it increased bone density in both ovariectomised (OVX) and sham operated mice. In addition it decreased bone levels of activin in OVX animals. Zoledronic acid, as expected, increased bone density in both OVX and sham animals via inhibition of osteoclast activity and caused a significant decrease in osteoblast numbers on the trabecular bone surface. Zoledronic acid decreased bone follistatin levels in OVX animals. *In vitro*, inhibin A prevented the zoledronic acid induced decrease in follistatin secretion from MDA-MB-231 cells.

These data suggest that addition of zoledronic acid to an inhibin A rich bone microenvironment with concurrently low activin levels, does not alter paracrine follistatin levels and therefore the low bioavailability of the tumour suppressor activin persists. This is in contrast to an inhibin A-deficient, high activin containing bone microenvironment (OVX), where zoledronic acid is able to decrease paracrine follistatin levels, potentially further increasing the bioavailability of activin. This could have implications for proliferation of DTCs within these two different bone environments. In addition to the modification of the bone microenvironment, inhibin A can directly affect breast cancer cells *in vitro,* causing an increase in follistatin secretion from MDA-MB-231 cells. These data suggest that the female hormone inhibin A can affect both the bone microenvironment and secretion of paracrine proteins from breast cancer cells and can potentially modify the response to zoledronic acid.

6.2 Introduction

The direct effects of the two members of the TGFβ superfamily of paracrine proteins, activin and follistatin, on breast cancer cells, have been extensively reported and are discussed in previous chapters. However, the direct effect of the TGFβ superfamily endocrine protein, inhibin, on breast cancer cells is not well defined in the literature. Sanz-Pamlona *et al* identified inhibin as a protein expressed in primary breast cancers, which predicted for formation of brain metastases (ROC AUC=0.91, 95% CI 0.11-1.00) (Sanz-Pamplona, Aragues *et al.* 2011), suggesting a metastasis promoting action of inhibin. These data were supported in a further study of multiple primary tumours including ovarian, colorectal and breast, which found inhibin A to be overexpressed in tumour samples, and predictive for a stage of tumourigenesis that corresponded with the ability of the tumours to invade stromal tissue and metastasise (Kim, Watkinson *et al.* 2010). Inhibin A has been shown to be over expressed in breast cancer cell lines compared to normal breast cell lines *in vitro* (Liang, Huuskonen *et al.* 2009), and an *in vivo* study of 334 breast cancer specimens found that inhibin A was expressed in 25% of invasive ductal carcinomas, 26% of DCIS and 33% of lobular carcinomas (Chang, Lee *et al.* 2005). However, other studies have found the opposite, with inhibin A expression being unchanged between normal breast tissue and invasive cancer (Reinholz, Iturria *et al.* 2002), or upregulated in normal breast tissue compared to invasive cancer (Di Loreto, Reis *et al.* 1999). The role of inhibin A in breast cancer therefore remains to be defined.

In early breast cancer, without clinically identifiable metastatic disease, disseminated tumour cells (DTCs) can be detected in the bone marrow in 30-40% of patients. The presence of DTCs are an independent adverse prognostic factor associated with an increased overall rate of death from breast cancer and decreased disease free interval (Braun, Vogl *et al.* 2005). The survival and fate of these DTCs is in part determined by their intrinsic properties, but is also influenced by cells in their surrounding environments including; bone cells (osteoblasts and osteoclasts), hematopoetic stem cells, endothelial stem cells, fibroblasts and immune cells. DTCs in bone are thought to interact with osteoblasts and compete for occupancy in the bone marrow (Park, Soki *et al.* 2011). In a murine prostate cancer model, cancer cells shed from sub-cutaneous tumours are demonstrated to occupy the hematopoetic stem cell niche, and expanding the niche by increasing osteoblast numbers with parathyroid hormone (PTH) increased the number of tumour cells in the bone marrow (Shiozawa, Pedersen *et al.* 2011). In a murine model of breast cancer, increasing osteoblast numbers and activity with PTH prior to intracardiac injection of MDA-MB-231 breast cancer cells resulted in an increased number of tumour foci (Brown HK 2013). In addition, tumour cells in direct contact with bone can alter bone cell numbers by decreasing osteoblast and increasing osteoclast numbers (Brown, Ottewell *et al.* 2012). These data support the concept that cells of the bone microenvironment are influenced by tumour cells, and can themselves affect tumour cell survival. The bone microenvironment will be altered by local paracrine factors but also by endocrine factors, including female hormones (Wilson, Holen *et al.* 2012). As discussed in chapter 3, premenopausal women have high inhibin levels compared to postmenopausal women, where inhibin levels are undetectable in serum. This could create two different bone microenvironments with the potential to differentially alter tumour cell colonization, growth and progression.

It is well established that activin is produced locally in the bone marrow and stored in the bone matrix (Ogawa, Schmidt *et al.* 1992), whereas inhibin is not abundantly expressed in bone. As discussed in chapter 5, inhibin's mechanism of action is via prevention of activin binding to its type 2 receptor, ActRIIA. Both *in vitro* and *in vivo* studies have investigated the effects of blockade of ActRIIA. *In vitro*, blockade of ActRIIA with inhibin A suppressed osteoblast and osteoclast formation and differentiation from murine BM cultures (Gaddy-Kurten, Coker *et al.* 2002), human mesenchymal stem cells and peripheral blood mononuclear cells (Perrien, Akel *et al.* 2007). In addition, despite secretion of activin from these cultures, the inhibitory effects of inhibin A on the ActRIIA receptor dominated.

Radiolabelled inhibin A administered intravenously was shown to accumulate within 10 minutes in the bone marrow of 25 day old rats, indicating that inhibin A can distribute to, and may have an effect on bone (Woodruff, Krummen *et al.* 1993). Three key *in vivo* studies (Perrien, Akel *et al.* 2007; Pearsall, Canalis *et al.* 2008; Chantry, Heath *et al.* 2010) have investigated the effect of blocking ActRIIA on bone, either with inhibin A or a soluble extracellular domain of ActRIIA fused to a murine IgG2a-Fc. These studies have collectively demonstrated that blocking this receptor increases bone density in mouse models (see Table 6.1), and the effect appears to be via an increase in the activity and number of osteoblasts (Ob).

These data suggest that the inhibin A/activin ratio in bone can determine the activity and number of osteoblasts with a subsequent effect on bone density. It could therefore be hypothesized that the bone microenvironment in an inhibin A rich (premenopausal) woman is different to that of an inhibin A deficient (postmenopausal) woman, and this may have subsequent effects on tumour growth/development (Fig 6.1)

The effect of zoledronic acid on bone density and cellular composition of bone has been extensively researched in both preclinical and clinical studies. Bisphosphonates bind avidly to bone and are taken up by osteoclasts (Coxon, Thompson *et al*. 2008). This leads to an inhibition of osteoclast mediated bone resorption in animal models, and can prevent bone loss in male and female animals who have or have not undergone OVX/gonadectomy (Gasser, Ingold *et al*. 2008) (Hornby, Evans *et al*. 2003). Maintenance of bone density does not therefore appear to be dependent upon sex or the hormonal status.

How the osteoclast-mediated effect of zoledronic acid subsequently modifies tumor cells is not clearly defined, but the general consensus is that tumour cells in bone may potentially be exposed to significant concentrations of bisphosphonates during bone turnover and hence be directly affected by the drug (Holen and Coleman 2010). An *in vivo* study of the effects of zoledronic acid on B16 melanoma tumour growth in bone in mice that lacked functional osteoclasts, still showed a decrease in tumour growth with zoledronic acid (Hirbe, Roelofs *et al*. 2009), suggesting the osteoclast may not be the only bone cell that influences the anti-tumour effects of zoledronic acid. The differential anti-tumour effects of zoledronic acid

Table 6.1 Summary of the main *in vivo* studies assessing the effect on bone of blockade of ActRIIA.

Mouse strain, sex and age at treatment	Treatment schedule	Duration of Treatment (weeks)	BV/TV	Osteoblast activity and number (trabecular surface)	Osteoclast activity and number (trabecular surface)	Ref
Transgenic Female intact Male sham/ ORCH 22 week	MFP induced inhibin A (Serum inhibin A levels 400-800 pg/ ml)	4	Proximal tibia; Treated vs control; F in tact;17% vs 10%*, M sham;35% vs 25%* M ORCH; 25% vs 15%*	F;NR M; increase in serum osteocalcin in sham*, and ORCH NS. No change in ObN in sham or orch	F;NR M; no change in serum CTx, or OcN in sham or ORCH.	Perrien *et al* (2007)
C57/BL/6N Female intact 12 week	ActRIIA-mFc 10mg/kg IP 2x per week	2,4,6,12	Distal femur; Treated vs control 2/52; 10% vs7%* 4/52; 15% vs6.5%* 6/52; 14% vs 6%* 12/52; 15% vs 4%*	2/52 increase in serum osteocalcin in treated vs control* 4/52 increase in serum osteocalcin in treated vs control* ObN; NR	2/52; no change in serum TRAP intreated vs control 4/52; increase in serum TRAP in treated vs control* OcN; NR	Pearsall *et al* (2008)
Female sham/ OVX 12 week		12	Distal femur ; HIgher in treated vs control in sham and ovx animals*	NR	NR	
C57/BL/6 Female intact 8 weeks	ACTRIIA-mFC, 0.1,0.3,3.0, 10mg/kg IP 2x per week	6	Proximal tibia; Dose dependent increase in BV/TV, 10mg/kg 22% vs control 9%	Increased ObN treated vs control*	No significant effect OcN treated vs control.	Chantry *et al* (2010)

Key; F female, M male, OVX ovariectomy, ORCH orchidectomy, IP intraperitoneal, * p value <0.05, NS not significant, ObN osteoblast number, OcN osteoclast number, NR not recorded, TRAP tartrate resistant acid phosphatase, CTx c terminal telopeptide, MFP=mifepristone.

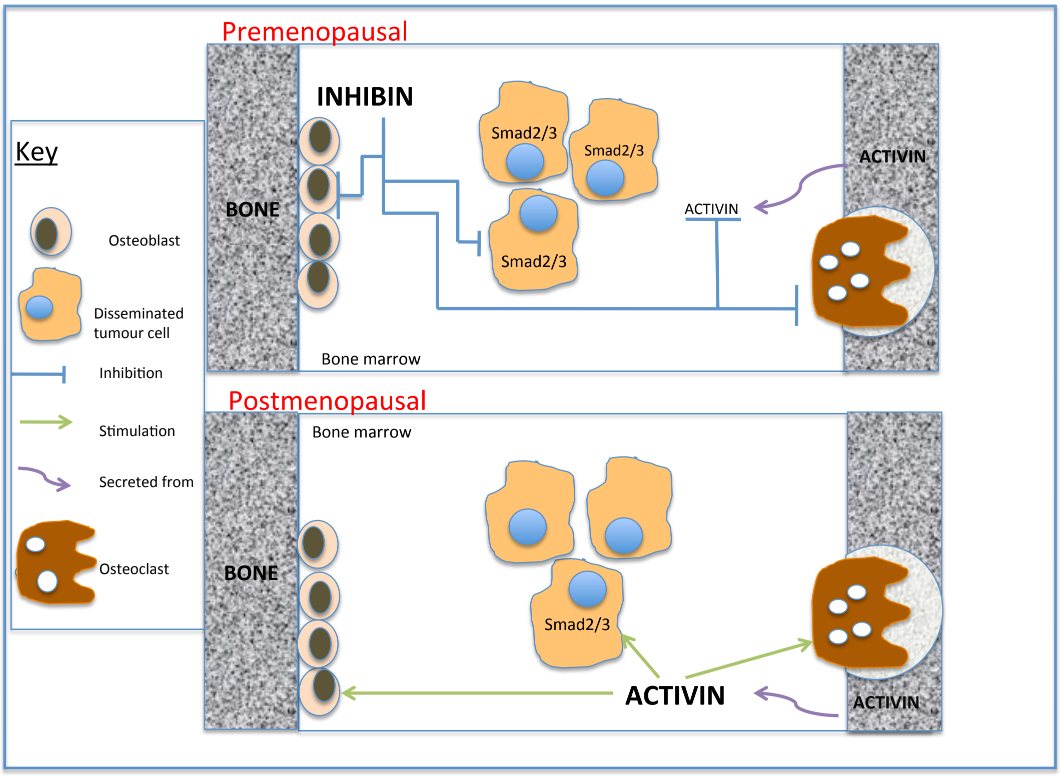


Figure 6.1. The bone microenvironment in premenopausal women will differ from that of postmenopausal women.

Premenopausal women have high levels of ovarian secreted inhibin A, which may have relevant effects on disseminated tumour cells within bone, by decreasing the ability of activin to bind to ActRIIA and phosphorylate the downstream signaling tumour suppressors Smad2/3. A postmenopausal woman has undetectable inhibin A due to natural ovarian failure, and therefore is likely to have a high level of paracrine secreted activin in bone that may influence disseminated tumour cells.

according to menopausal status seen in the clinical trials and discussed in chapters 3 and 4 may therefore, in part, be due to mechanisms of action other than inhibition of bone resorption, since the inhibition of osteoclast activity is not a sex or hormone-mediated mechanism of action of zoledronic acid (Hadji, Coleman *et al*. 2012).

There is very little published data on the effect of bisphosphonates on activin levels or release from bone. Sakai *et al* reported that activin was released from mouse calvaria into medium in response to parathyroid-induced bone resorption, and that addition of a bisphosphonate (disodium dichlormethane-1,1-bisphosphonic acid) inhibited the release of activin from bone (Sakai, Eto *et al*. 2000). This effect was not seen in primary culture of calvarial cells, suggesting the mechanism of action was via release of activin from bone during resorption, rather than direct secretion of activin from bone cells.

To my knowledge there are no published *in vivo* studies evaluating the effect of either inhibin A or zoledronic acid on activin or follistatin release from bone, or the effect of inhibin A on follistatin secretion from breast cancer cells *in vitro*. The new information described in this chapter details how inhibin A and zoledronic acid differentially affect activin and follistatin release in both the bone microenvironment *in vivo,* and directly from MDA-MB-231 cells *in vitro*. These data suggest a novel opposing action of the female hormone inhibin A and zoledronic acid on the activin:follistatin ratio *in vivo* and *in vitro*, with potential implications for development of bone metastases.

6.3 Aims

The overall purpose of this chapter was to evaluate the effect of inhibin A and zoledronic acid on activin and follistatin levels in the bone microenvironment *in vivo*, and the implications for breast tumour cell growth. The following aims were set:

1. To establish if inhibin A can alter secretion of activin or follistatin from breast cancer cells, and the interaction with zoledronic acid *in vitro.*

2. To establish the tolerability and biological activity *in vivo* of a recombinant human inhibin A protein.

3. To establish if recombinant human inhibin A can prevent ovariectomy-induced bone loss *in vivo.*

4. To determine if inhibin A alters the level of activin and follistatin in the bone and liver in ovariectomised and sham operated mice.

5. To confirm the effects of zoledronic acid on the bone microenvironment in ovariectomised and sham-operated mice, and to evaluate if zoledronic acid alters the level of activin and follistatin in the bone and liver microenvironment.

6.4. Materials and methods

See materials and methods chapter 2 for further information.

Unless stated otherwise, all *in vitro* experiments were carried out with 3 replicates and 3 repeats.

6.4.1 The effect of inhibin A on paracrine secretion of activin and follistatin in ER-ve and ER+ve breast cancer cells, and the interaction with zoledronic acid.

Two cell lines were used, the ER-ve MDA-MB-231 and the ER+ve MCF7. Cells were seeded in 6 well plates as detailed in chapter 2, at densities chosen to reflect specific cell line rate of replication, with an aim to achieve ~80% confluence of cells at the end of the experiment. Cells were maintained in DMEM+10%FCS for 48 hours with or without 10ng/ml inhibin A. Live cell count was performed from each well at the end of the experiment, to enable normalization of ELISA level to cell count by expressing results as secretion/million cells. Supernatant was collected at 48 hours and processed to activin and follistatin ELISA to quantify secreted levels of each protein. Further secretion experiments were performed in MDA-MB-231 cells with addition of 10ng/ml inhibin A +/- 50μM zoledronic acid compared to medium only control. To evaluate the effect of conditioned medium from zoledronic acid and inhibin A treated MDA-MB-231 cells, a fluorescent cell based immunoassay was used to quantify levels of pSmad2/3 relative to total Smad2/3 in MDA-MB-231 cells treated for 1 hour with conditioned medium.

TGFβ secretion in response to 10ng/ml inhibin A was assessed by removing supernatant from control and inhibin A treated MDA-MB-231 cells at 48 hours and processing to TGFβ1 ELISA with levels normalized to cell count.

6.4.2. To establish the tolerability and biological activity *in vivo* of a recombinant human inhibin A protein sourced from NIBCS.

Sub-cutaneous osmotic pumps were inserted into 12-week old balb/c nude female mice and primed to deliver control vehicle (1xPBS), 10ng/day inhibin A or 60ng/day inhibin A for 28 days. Details of surgical procedure are found in chapter 2. To establish tolerability weight was recorded and liver was fixed in 4% PFA for 24 hours and then processed to wax and sectioned for H&E stain. To evaluate biological activity, blood was collected at termination of experiment for serum measurement of follicle stimulating hormone (FSH), and markers of bone resorption (TRAP) and bone formation (P1NP), and right hind legs were fixed in 4% PFA, imaged using μCT software and subsequently decalcified for 2 weeks prior to processing to wax and sectioning for bone histomorphometry.

6.4.3 To establish if recombinant human inhibin A can prevent ovariectomy induced bone loss *in vivo.*

12-week old balb/c nude female mice underwent ovariectomy or sham operation as detailed in chapter 2. During the same surgical procedure, sub-cutaneous ALZET osmotic pumps were inserted having been primed to deliver control vehicle (1xPBS) or 60ng/day inhibin A for 28 days. At termination of experiment, cardiac blood was collected and serum was processed for human inhibin A, mouse TRAP and P1NP ELISA. Right hind limbs were processed as described in 6.4.2.

6.4.4 To determine if inhibin A alters the level of activin and follistatin in the bone and liver in ovariectomised and sham operated mice.

Liver and right hind limbs were processed from animals undergoing the procedure detailed in 6.4.3. Liver was homogenized in 9mls of 1xPBS, centrifuged and supernatant processed to mouse activin and follistatin ELISA normalized to liver weight.

Calvaria were processed as per chapter 2; briefly they were crushed in 2.5mls PBS, filtered and then centrifuged. Supernatant was removed and cell count was performed using trypan blue. Supernatant was processed to activin and follistatin ELISA normalized to cell count.

6.4.5 To determine if zoledronic acid alters the level of activin and follistatin in the bone microenvironment and liver in ovariectomised and sham-operated mice.

12-week old balb/c nude mice were treated for 4 weeks with weekly IP zoledronic acid 100μg/kg staring on day 1 of experiment. Mice underwent ovariectomy or sham operation on day 2 of the experiment. At termination of the experiment, cardiac blood was collected and serum was processed to mouse TRAP and P1NP ELISA.

The right hind limb, liver and calvaria were collected and processed as described in 6.4.3 and 6.4.4

6.5 Results

6.5.1 The effect of inhibin A on secretion of activin and follistatin in ER-ve and ER+ve breast cancer cells, and the interaction with zoledronic acid.

To determine if inhibin A can alter the secretion of either activin or follistatin from MDA-MB-231 and MCF7 cells, cells were exposed to media alone or media plus 10ng/ml of inhibin A for 48 hours. In MDA-MB-231 cells inhibin A significantly increased secretion of activin compared to control (p value 0.0357), and although inhibin A increased follistatin secretion by 44%, this did not reach statistical significance. MCF7 cells showed no alteration in their secretion of activin or follistatin with the addition of inhibin A (Fig 6.2).

To determine if inhibin A can affect the changes in follistatin seen with zoledronic acid in ER-ve breast cancer cells, MDA-MB-231 cells were exposed to either medium alone, medium plus 10ng/ml inhibin A +/- 50μM zoledronic acid for 48 hours. The significant fall in follistatin secretion with zoledronic acid compared to control was lost with the addition of inhibin A, which increased follistatin levels by 30% compared to zoledronic acid alone (Fig 6.3).

These data showed that inhibin A increases activin secretion from MDA-MB-231 cells, but that follistatin secretion is also increased. This reduces the net effect of zoledronic acid on decreasing levels of follistatin. To evaluate if these changes in protein secretion could influence the downstream phosphorylation of Smad2/3C, conditioned media from MDA-MB-231 cells treated for 48 hours with either medium alone or 50μM zoledronic acid +/- 10ng/ml inhibin A was added to MDA-MB-231 cells for 1 hour, in a cell based immunoassay (Fig 6.4). MDA-MB-231 cells treated with supernatant from zoledronic acid treated cells had a significantly higher percentage of pSmad2/3C to total Smad2/3C compared to all other groups (p=0.05). Direct exposure of MDA-MB-231 cells to either 50μM ZOL or 10ng/ml inhibin A for 1 hour had no significant effect on pSmad2/3C levels (Fig 6.4), indicating that the changes in follistatin secretion into supernatant from MDA-MB-231 cells treated with zoledronic acid and inhibin A was driving the changes in pSmad2/3C, rather than the direct effect of the hormone/drug themselves.

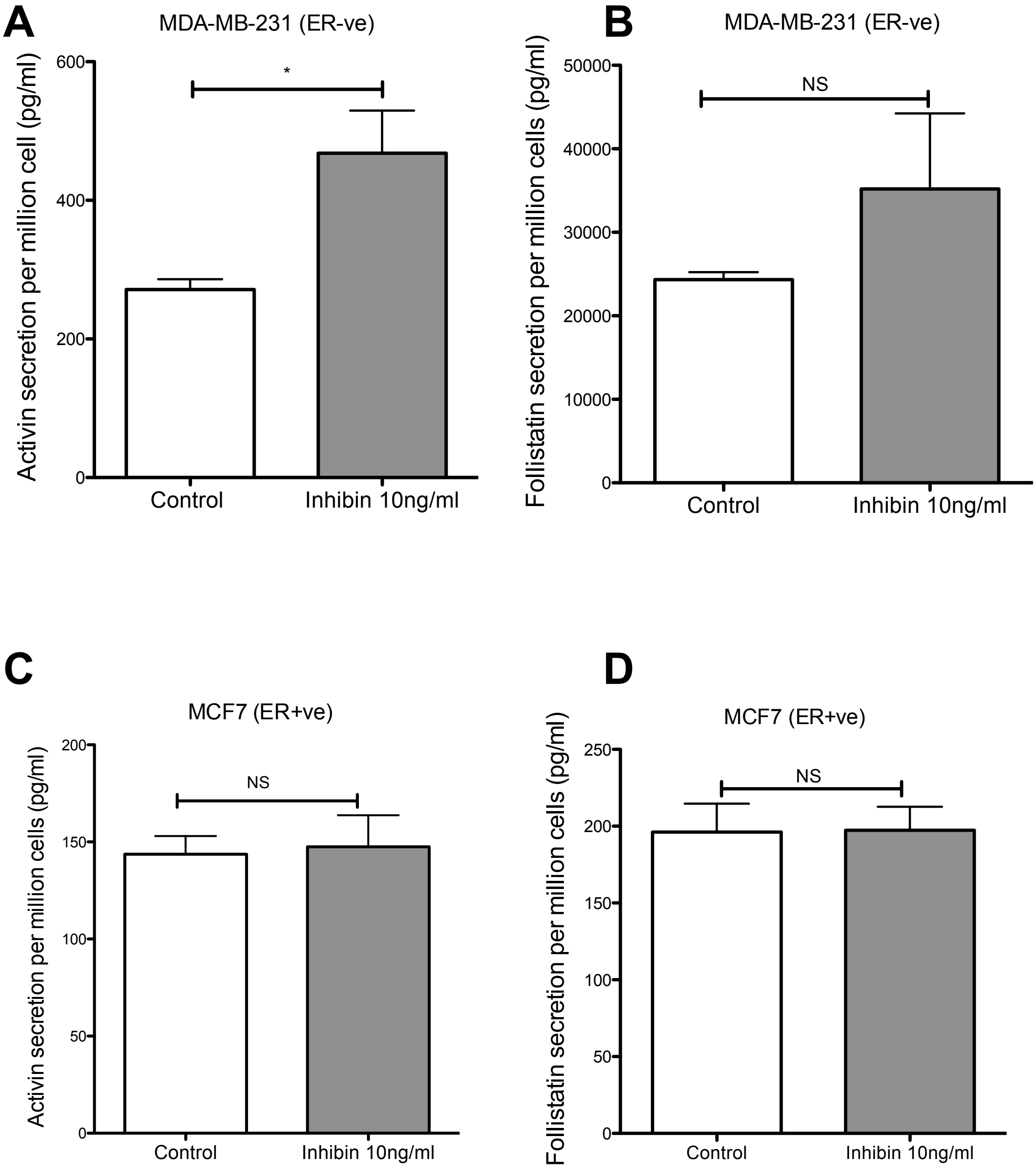


Figure 6.2 The effect of inhibin A on secretion of activin and follistatin from MDA-MB-231 and MCF7 cells.

MDA-MB-231 (A,B) and MCF7 (C,D) breast cancer cells were seeded in 6 well plates and maintained for 48 hours in either medium alone or medium+10ng/ml inhibin A. At 48 hours the supernatant was collected and processed to activin or follistatin ELISA and values normalized to cell count. Data represent mean +SEM of 3 replicates and 3 repeats. Mann Whitney test for significance, * p value <0.05.

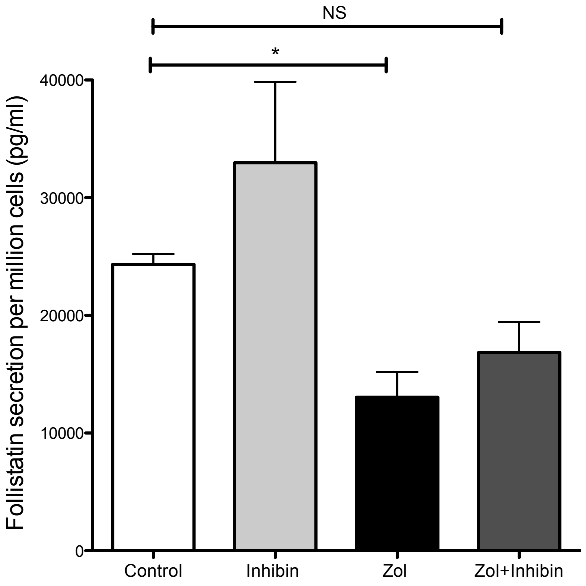


Figure 6.3 Secretion of follistatin from ER-ve MDA-MB-231 in response to inhibin A and zoledronic acid.

Cells were seeded in 6 well plates and exposed for 48 hours to medium alone +/- 50μM zoledronic acid and/or 10ng/ml inhibin A. At 48 hours supernatant was processed to follistatin ELISA and values normalized to cell count. Data represent mean +SEM of 3 replicates and 3 repeats, Mann Whitney test for significance, *p value <0.05.

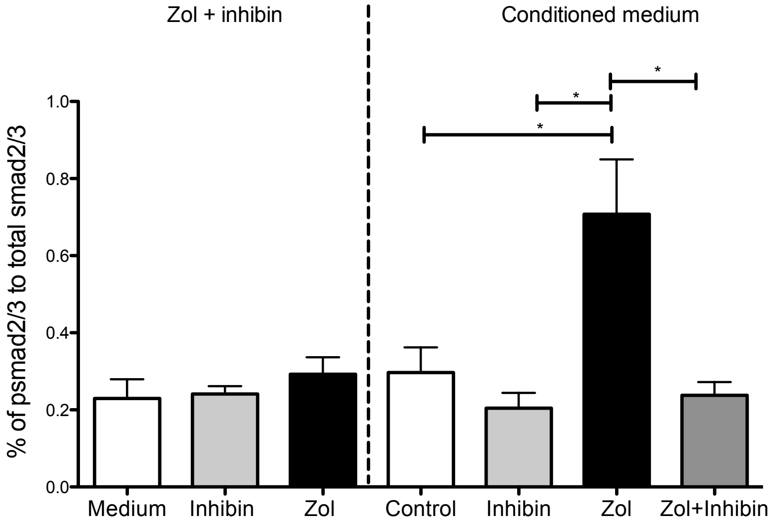


Figure 6.4 Effect of supernatant from inhibin A and zoledronic acid treated MDA-MB-231 cells on pSmad2/3 levels in MDA-MB-231 breast cancer cells.

Cells were seeded per well in a 96 well plate and exposed to 1 hour of conditioned medium from MDA-MB-231 cells previously treated for 48 hours with 10ng/ml inhibin A +/- 50μM zoledronic acid (Fig 6.3). MDA-MB-231 cells were also exposed to 1 hour of 10ng/ml inhibin A and 50μM zoledronic acid. Results are expressed as % of pSmad2/3 to total Smad2/3. Data represents mean+SEM of percentages from 3 replicates of 3 repeated experiments, Mann Whitney test for significance, * p value <0.05

As discussed in chapter 5, Smad2/3 is phosphorylated in response to both activin and TGFβ binding to cell surface receptors. Previous data (see Fig 5.14) showed that zoledronic acid does not significantly alter TGFβ secretion from MDA-MB-231 cells. To ensure that the changes in pSmad2/3 seen with addition of inhibin A to zoledronic acid were due to alterations in the activin pathway, a further experiment was done to determine if inhibin A affected TGβ secretion from MDA-MB-231 cells. Medium was collected after 48 hours of exposure to medium+/-10ng/ml inhibin A and processed to TGFβ1 ELISA with values normalized to cell count. Inhibin A did not significantly alter TGFβ1 secretion from MDA-MB-231 cells after 48 hours (Fig 6.5).

These data show that inhibin A decreases the inhibitory effect zoledronic acid has on follistatin secretion from MDA-MB-231 cells, with subsequent reduction in phosphorylation of the intracellular Smad 2/3 proteins downstream of the ActRII receptor.

6.5.2 To establish the tolerability and biological activity *in vivo* of a recombinant human inhibin A protein sourced from NIBCS.

Recombinant inhibin A has not been commercially available for several years since the major supplier (Beckman Coulter) stopped producing the ligand (personal communication with Professor Gaddy, University of Arkansas, Texas). A potential source of inhibin A was identified from the suppliers of the World Health Organisation (WHO) standard inhibin A (NIBSC). The concern at this stage was that inhibin A preparations can be contaminated with activin A, which will affect *in vivo* results. After discussion with the WHO suppliers and advice from Professor Gaddy, a small *in vivo* pilot study was undertaken to evaluate if the recombinant protein was biologically active, as assessed by measuring follicle stimulating hormone levels and evaluation of the effects on bone in 12-week old female balb/c nude mice. To establish the expected physiological levels of inhibin A from mice of this age, serum from a previous *in vivo* experiment (carried out by Dr Penny Ottewell) was used. Mice were treated with weekly intra-peritoneal (IP) saline or 100mg/kg zoledronic acid after ovariectomy (OVX) or sham operation for 28 days. Serum previously collected and stored at -80°C was thawed and analysed using a mouse specific inhibin A ELISA. The mean serum level of inhibin A from mice that had undergone OVX was not statistically different from sham-operated mice (Fig 6.6). The physiological level of inhibin A from the control mice was then used to calculate planned

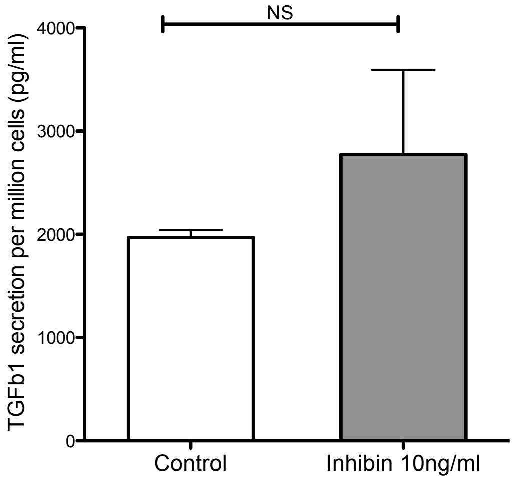


Figure 6.5 TGFβ1 secretion from MDA-MB-231 cells in response to inhibin A.

Cell were seeded in 6 well plates and exposed for 48 hours to medium alone or medium+10ng/ml of inhibin A. After 48 hours the supernatant was removed and processed to TFGβ1 ELISA with values normalized to cell count. Data represents mean+SEM. Mann Whitney test for significance, NS = not significant

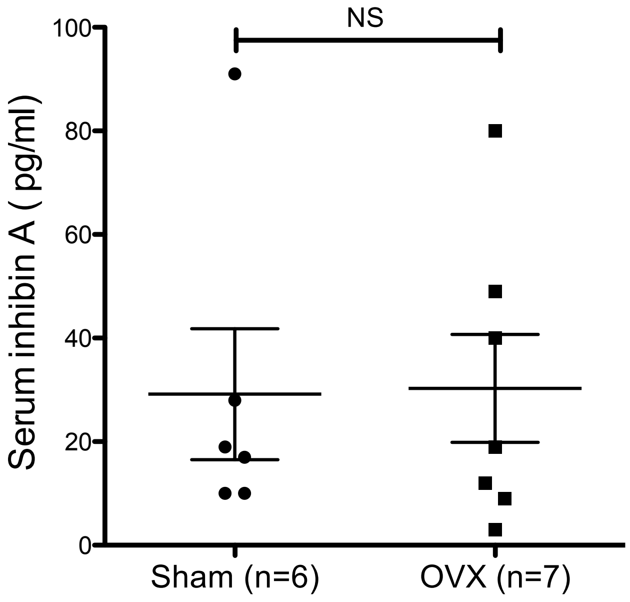


Figure 6.6. Serum inhibin A levels in 12-week old balb/c nude female mice following sham or ovariectomy.

Serum was collected at cull from female balb/c nude 12-week old female mice either ovariectomised or sham operated, that had been maintained for 6 weeks and treated with weekly IP saline injections. Dots represent serum values in sham animals, squares represent OVX animals. Mann Whitney test for significance, NS=not significant.

doses of recombinant inhibin A to add to the ALZET pumps.

To evaluate the effect of inhibin A *in vivo*, ALZET pumps were loaded with either 10ng/day inhibin A (n=3) or 60ng/day inhibin A (n=3) in addition to PBS vehicle control (n=3) (as described in section 2.2.3). 12-week old female balb/c nude mice were anaethetised and the sub-cutaneous pumps were inserted and wound closed with staples. Mice were maintained for 28 days and then sacrificed by cervical dislocation with collection and tissues were collected and processed as detailed in 6.4.2.

6.5.2.1 Assessment of tolerability to inhibin A

Tolerance was assessed by observing behaviour and general well-being of animals by monitoring the weight of the animals during the procedure, and evaluation of hepatic architecture at termination of experiment since inhibin A is metabolised by the liver. No abnormal behaviour was observed in the animals treated with either dose of inhibin A and no problems were encountered with wound healing or closure. Weights increased during the course of the experiment in all mice apart from a single animal in the 60ng/day group. This mouse was smaller than all others at baseline, and by day 9 had begun to lose weight (-0.4% compared to baseline) increasing to -6% at day 15 and -15% at day 26, and was therefore culled at this time-point and was excluded from further analysis. To evaluate gross hepatic toxicity, liver sections were stained with H&E. No difference was seen in the overall structure of liver sections from control or inhibin A treated mice indicating no severe hepatic toxicity resulted from treatment with the recombinant inhibin A (Fig 6.7).

6.5.2.2 Evaluation of the biological activity of recombinant inhibin A

To evaluate the biological activity of inhibin A, serum levels of FSH, P1NP and TRAP were measured and right hind leg was analysed for bone volume and effects on osteoblast/osteoclast numbers by histomorphometry.

Secretion of pituitary FSH is under negative feedback from both oestrogen and inhibin A (Welt, Pagan *et al*. 2003); therefore inhibin A would be predicted to decrease serum FSH. Serum FSH did not significantly differ between the treatment groups (Fig 6.8 A), this may reflect a lack of biological activity of the inhibin A, or may be due to timing of sample collection at day 28.

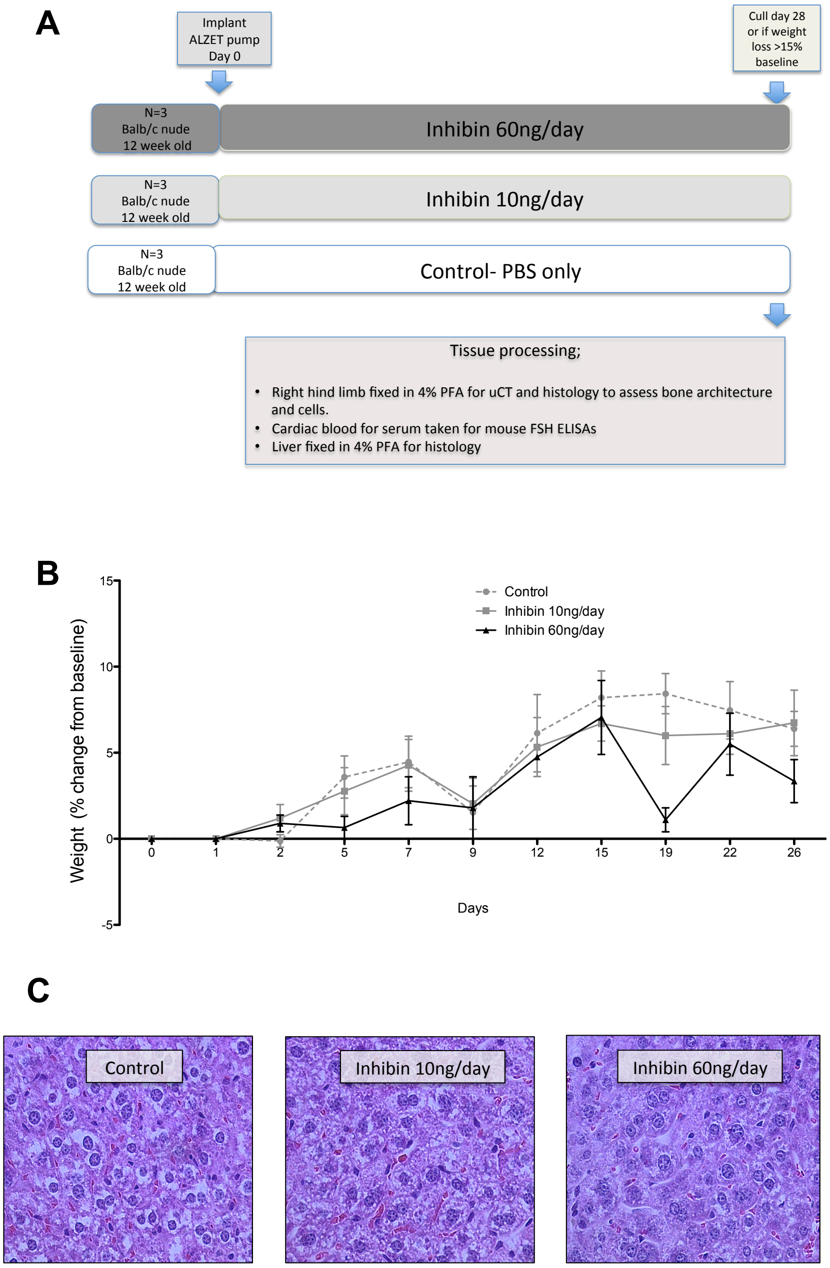


Figure 6.7 Evaluation of the toxicity of inhibin A *in vivo*.

A Experimental outline; animals were anaesthetized on day 0 and implanted with sub-cutaneous osmotic pumps primed to deliver vehicle only (control), inhibin A 10ng/day or 60ng/day. Mice were maintained for 28 days and evaluated for signs of toxicity from inhibin A. **B**; serial measurements of weight over duration of experiment, data represent mean +SEM percentage change from baseline weight over 28 days. **C;** Representative H&E stained sections of liver from control, inhibin A 10ng/day and inhibin A 60ng/day.

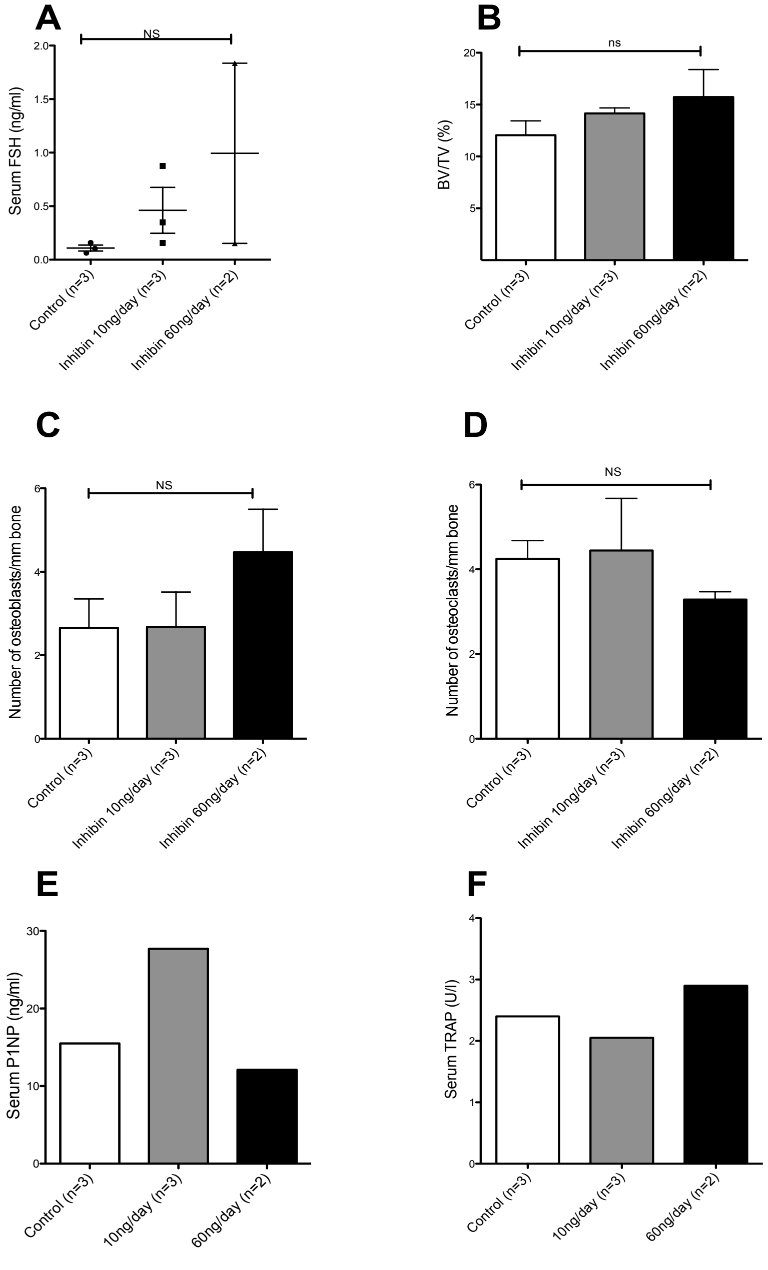


Figure 6.8 Assessment of biological activity of inhibin A *in vivo.*

Mice were treated for 28 days with continuous sub-cutaneous infusion of inhibin A via ALZET pump delivered at 10ng/day or 60ng/day dissolved in 1x PBS, or PBS control**. A**; serum FSH levels shown per animal and expressed as mean+SEM. **B** Bone volume:tissue volume (BV/TV) per treatment group. **C+D**; Osteoblast and osteoclast number per mm bone per treatment group. All data represent mean+SEM, student t test for significance, NS not significant. **E+F;** Serum P1NP as a marker of osteoblast activity, and serum TRAP as a marker of osteoclast activity from pooled serum from each treatment group.

Bone volume: tissue volume (BV/TV) did not differ significantly between groups although there was a consistent trend for an increase in BV/TV with increasing doses of inhibin A (Fig 6.8 B+6.9), this was reflected by a trend towards higher number of osteoblasts per mm of bone and a lower number of osteoclasts per mm of bone in the 60ng/day inhibin A group compared to control, although this did not reach statistical difference ($p=0.18$) (Fig 6.8 C+D and 6.9). There was no consistent trend in changes in the bone formation marker P1NP or bone resorption marker TRAP in pooled serum samples (Fig 6.8 E +F)

These data showed that the recombinant inhibin A protein was well tolerated *in vivo* and the method of delivery via osmotic pump was safe. The biological activity of the protein was inconclusive at this stage due to small numbers of animals per group, however, a trend to increasing bone: tissue volume was seen, with an associated rise in osteoblast number and a fall in osteoclast number. In order to evaluate the effect of inhibin A on the bone microenvironment in a larger cohort of mice, a second *in vivo* experiment was performed with 5 mice per group treated with the higher dose of inhibin A (60ng/day) or vehicle control, in both OVX and sham animals.

6.5.3 To establish if recombinant human inhibin A can prevent ovariectomy induced bone loss *in vivo.*

Intact animals (sham) and ovariectomised (OVX) were treated with 60ng/day inhibin A or PBS control for 28 days. Weights fell in all groups, except the sham inhibin A group for the first 11 days post-surgery, but then recovered to ≥ baseline. This is likely to reflect the invasiveness of the OVX/sham procedure, and use of opiates as analgesia at surgery affecting appetite (Fig 6.10)

6.5.3.1 Effects of 60ng/day inhibin A on bone.

To evaluate the effect of inhibin A on bone structure and bone cell numbers, the right proximal tibia was fixed in 4% PFA and scanned using μCT (as previously described in chapter 2). The tibia was then decalcified for 2 weeks and processed to wax for sectioning. 2 serial sections were stained for TRAP to identify osteoclasts, and the whole trabecular surface quantified using osteomeasure software.

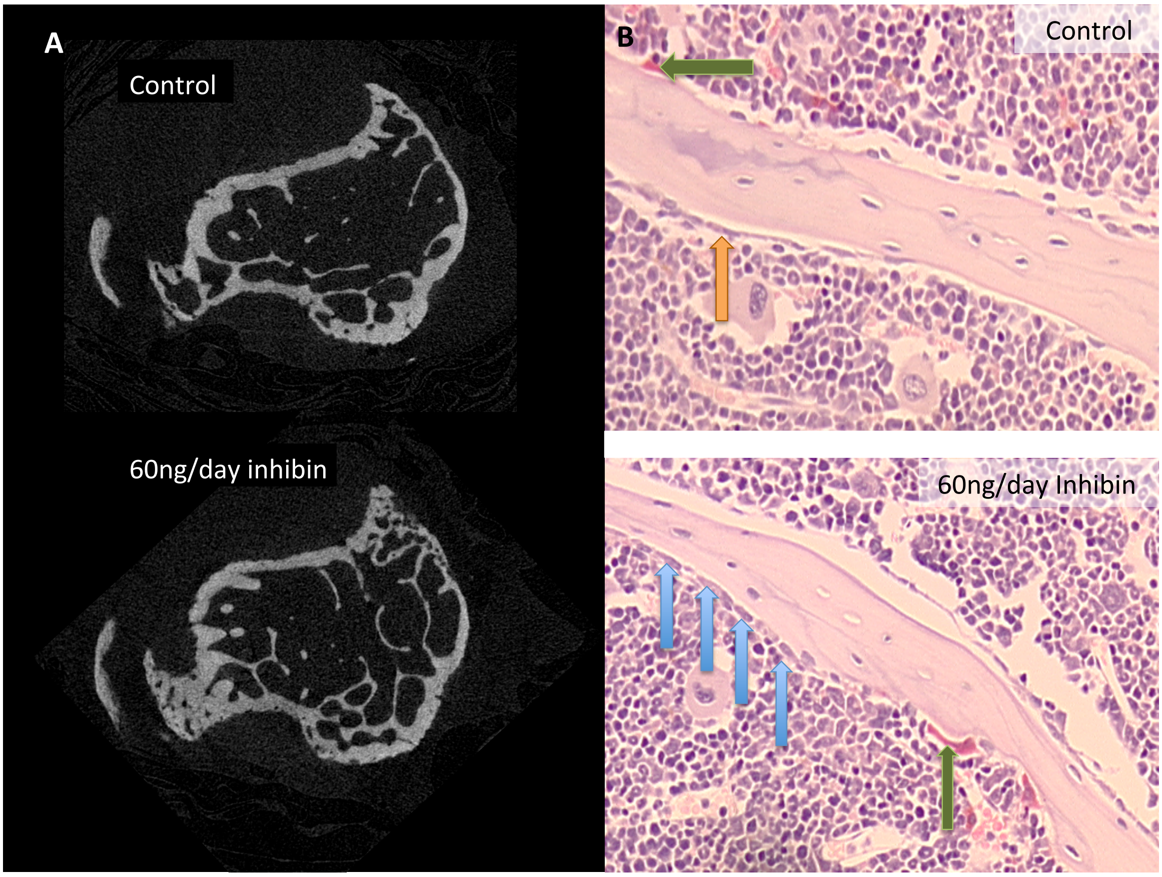


Figure 6.9. Representative μCT images and TRAP stained sections of proximal right tibia from mice treated with PBS or 60ng/day inhibin.

A; Representative cross sectional images of right hind leg were obtained using μCT in control and inhibin treated mice. **B;** Sections from right tibia were TRAP stained to allow identification of osteoblasts and osteoclasts on bone trabecular surface in control and inhibin treated mice, blue arrows represent osteoblasts, orange arrows represent bone-lining cells and green arrows represent osteoclasts.

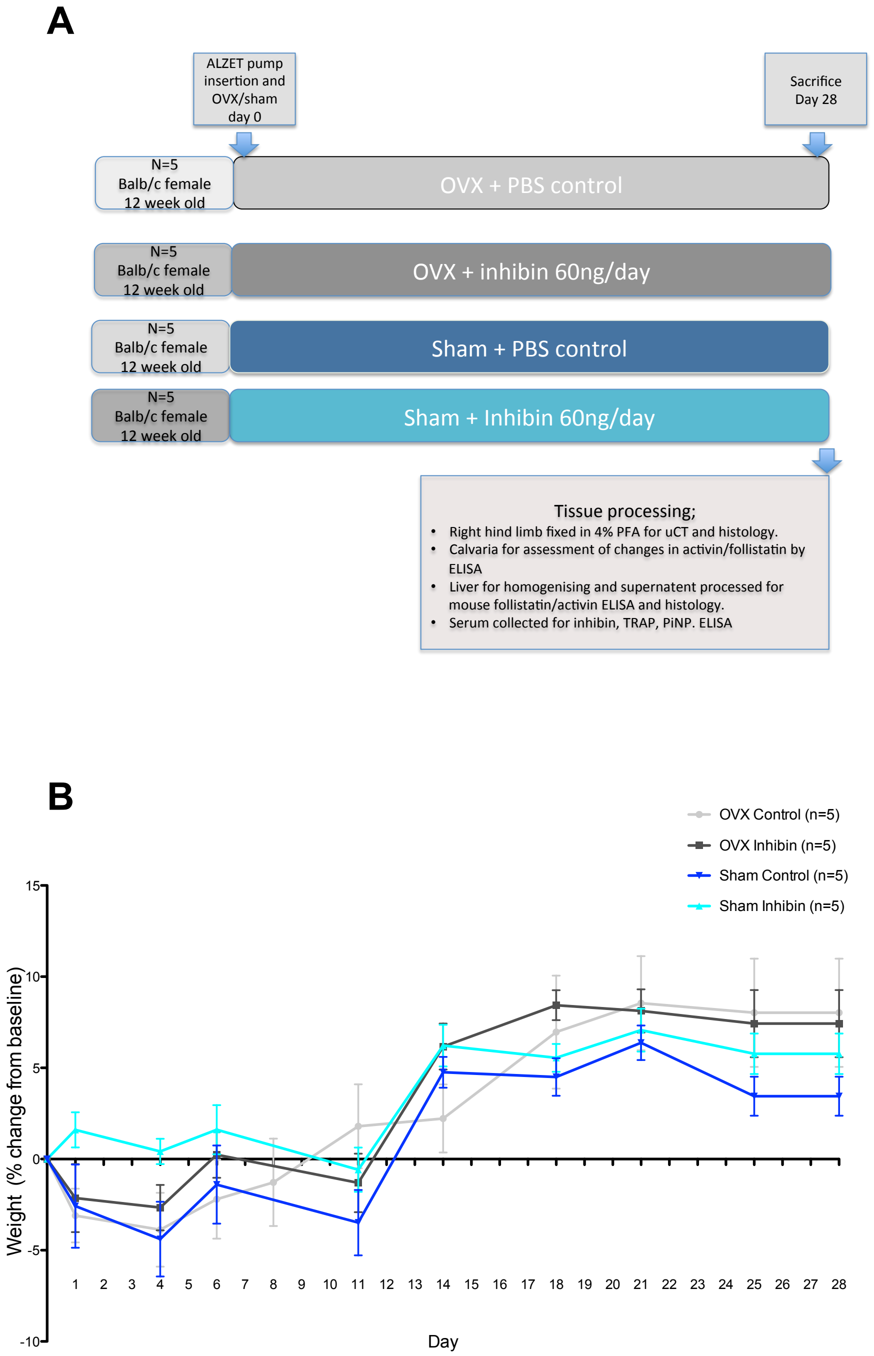


Figure 6.10. **Experimental outline to assess effect of inhibin A on the bone microenvironment in OVX and sham operated mice**

A; On day 0 animals underwent OVX (n=10) or sham (n=10) operation and were implanted with sub-cutaneous ALZET osmotic pumps primed to deliver PBS only or inhibin 60ng/day dissolved in PBS. Mice were maintained for 28 days with tissue collected for downstream analyses as outlined. **B;** Serial measurements of weight over duration of experiment, data represent mean +SEM percentage change from baseline weight over 28 days.

OVX induced bone volume loss compared to sham, and although this was partly reversed by inhibin A it did not reach statistical significance (p=0.2) (Fig 6.11a and 6.12 A). Inhibin A significantly increased bone volume compared to vehicle in sham-operated mice (p=0.0079). Bone cells were counted on TRAP stained sections allowing identification of osteoblasts and osteoclasts covering the trabecular bone surface of the proximal right tibia (Fig 6.11b and 6.12 B+C). OVX significantly decreased osteoblasts number and increased osteoclast number compared to sham. Inhibin A did not significantly alter the number of osteoblasts/mm bone compared to control in OVX or sham animals, however, there was a significant increase in osteoclasts/mm bone in sham operated mice receiving inhibin A compared to sham control (p=0.03).

Serum TRAP (a marker of osteoclast activity), and P1NP (a marker of osteoblast activity) was measured in pooled samples due to insufficient individual sample volumes for analysis. P1NP levels were higher in sham animals vs. OVX animals, and addition of inhibin A did not alter TRAP or P1NP in OVX or sham animals (Fig 6.12 D+E).

No significant difference was found in serum levels of inhibin A between control and inhibin A treated animals in either the OVX or sham group (p=0.3) (Fig 6.13).

Taken together, these results show that the inhibin A appeared to have relevant biological effects in bone by increasing BV/TV, however, this was only apparent in sham operated mice. Measurement of the serum inhibin A levels suggested that the dose of inhibin A delivered by the pumps was insufficient to cause an increase in serum inhibin A compared to physiological levels in control mice. In order to evaluate if a higher dose of inhibin A could induce effects on bone in OVX mice, the experiment was repeated with the osmotic pumps primed to deliver 120ng/day inhibin A.

6.5.3.2 Effects of 120ng/day inhibin A on bone.

The increased dose of inhibin A (120ng/day) was well tolerated, and although weights fell in all groups for the first 7 days post-surgery, it recovered to ≥ baseline (Fig 6.14), which was comparable to the effects seen with 60ng/day inhibin A

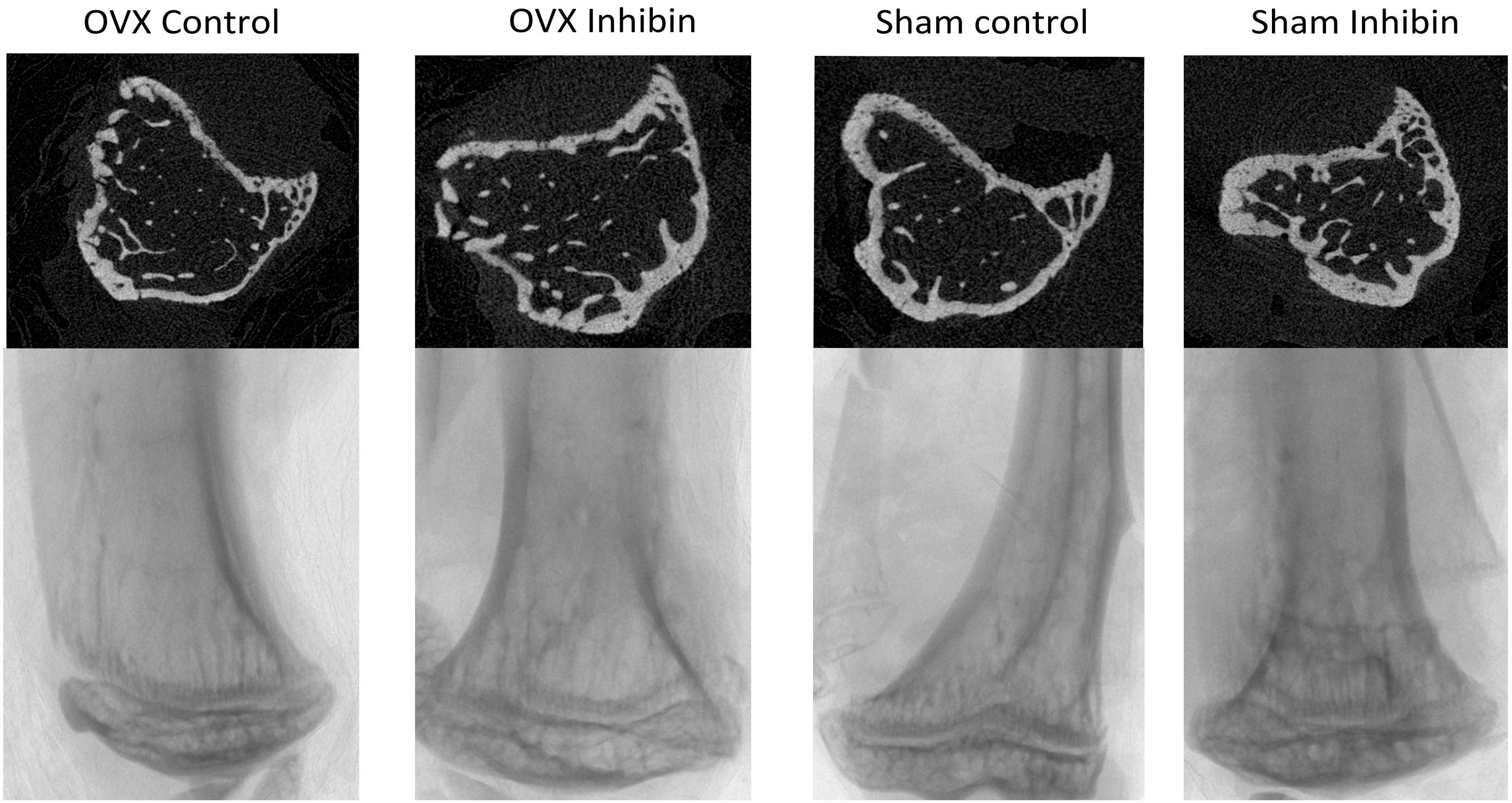


Figure 6.11a Effect of 60 ng/day inhibin A on bone in OVX and sham mice- representative μCT images of proximal right tibia

Representative μCT cross sectional images of the proximal right tibia (top) with associated radiographs (bottom) from OVX and sham mice treated with and without 60ng/day.

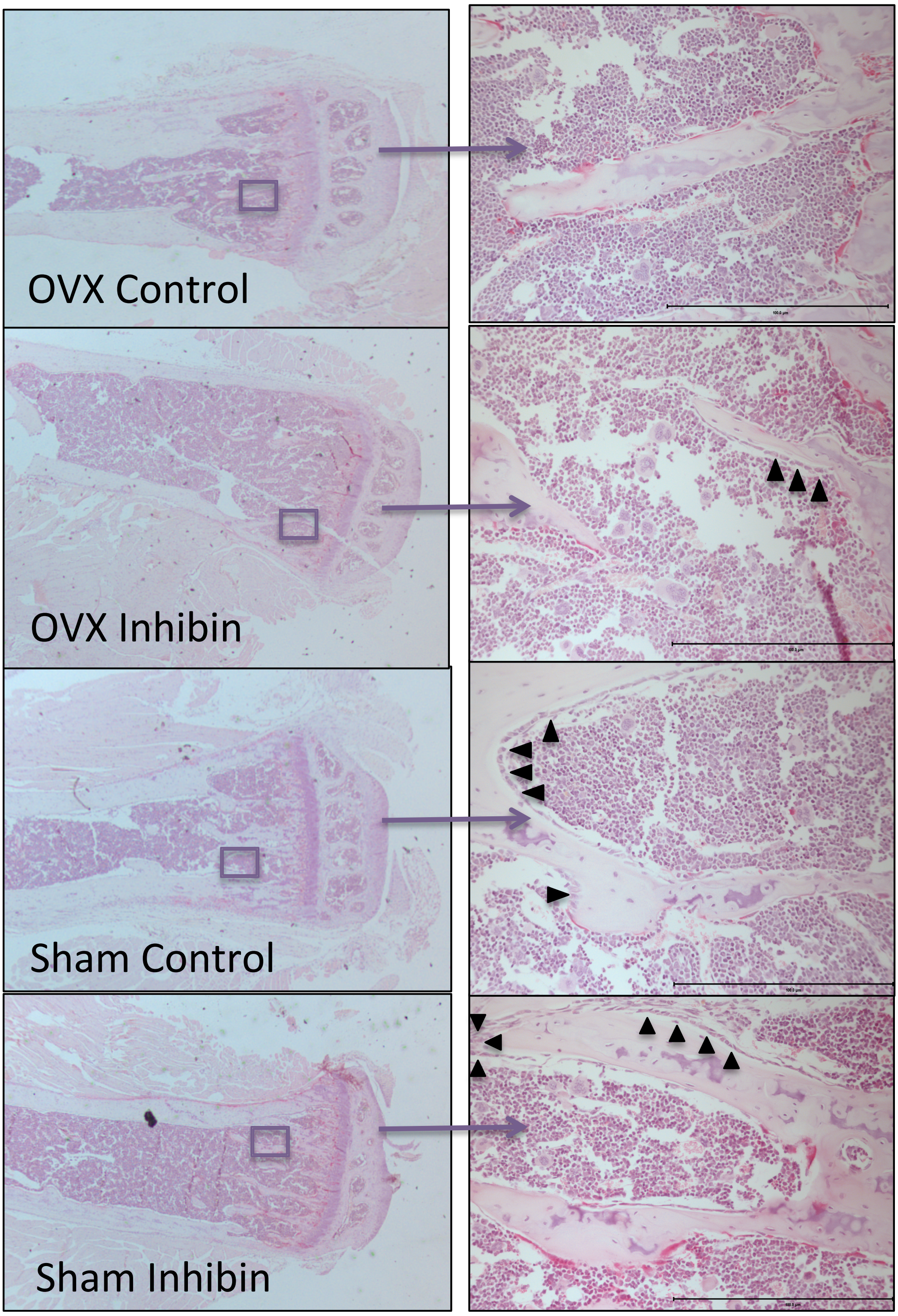


Figure 6.11b Effect of 60ng/day inhibin A on osteoblasts and osteoclasts in OVX and sham mice -representative TRAP stained sections in OVX and sham mice

Arrowheads represent osteoblasts sitting on the surface of the bone. Purple box represents the area of magnification increased from x1.6 (left) to x20 (right). Scale bar represents 100μm.

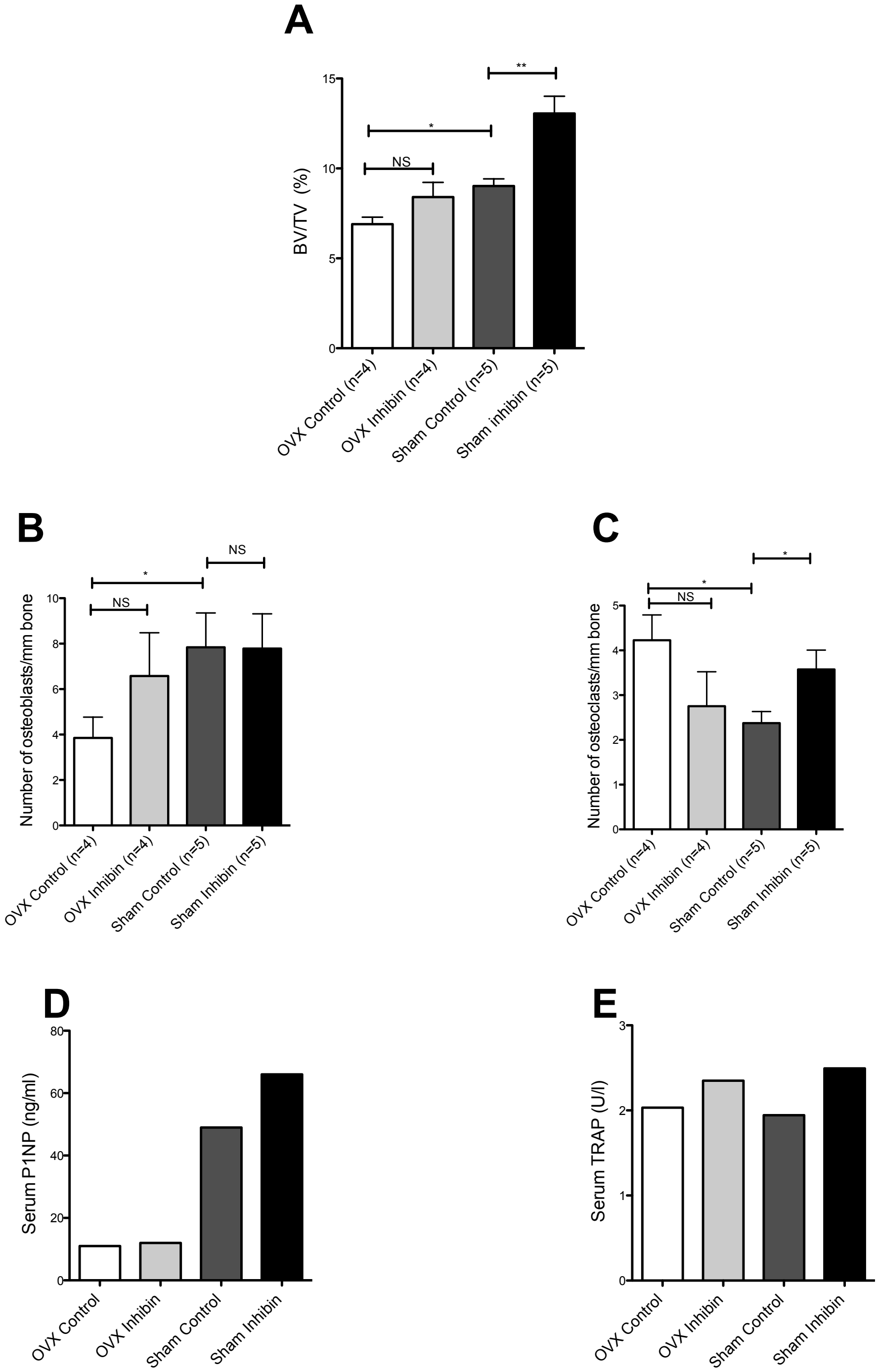


Figure 6.12. Effect of 60ng/day inhibin A on bone volume, osteoblast and osteoclast number and activity in animals following OVX or sham operation.

Mice were treated for 28 days with continuous sub-cutaneous infusion of 60ng/day inhibin A via ALZET pump, or PBS control, post OVX or sham operation. **A**; BV/TV in OVX and sham animals assessed using μCT imaging. **B+C;** Osteoblast and osteoclast number per mm bone quantified using TRAP stained sections. **D+E** Serum P1NP as a marker of osteoblast activity and serum TRAP as a marker of osteoclast activity. Data **A-C** represent mean +SEM, Mann Whitney U test for significance, ** p value <0.01, * p value <0.05, NS not significant. Data **D+E** represent pooled serum samples from each group therefore no further statistical analysis was possible.

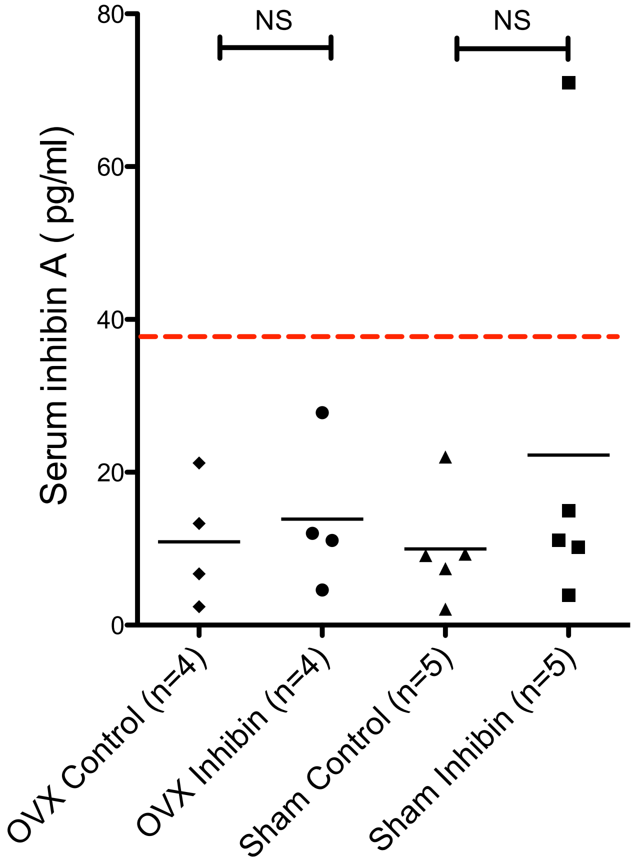


Figure 6.13. Serum levels of human inhibin A in mice treated with and without 60ng/day inhibin A following OVX or sham operation.

Mice were treated for 28 days with continuous sub-cutaneous infusion of 60ng/day inhibin A via ALZET pump, or PBS control, post OVX or sham operation.
Serum was collected at the end of experiment and processed for human inhibin A ELISA. Dots represent values from individual animals. Mann Whitney test for significance. NS=not significant.
Red line represents lower limit of detection for assay used.

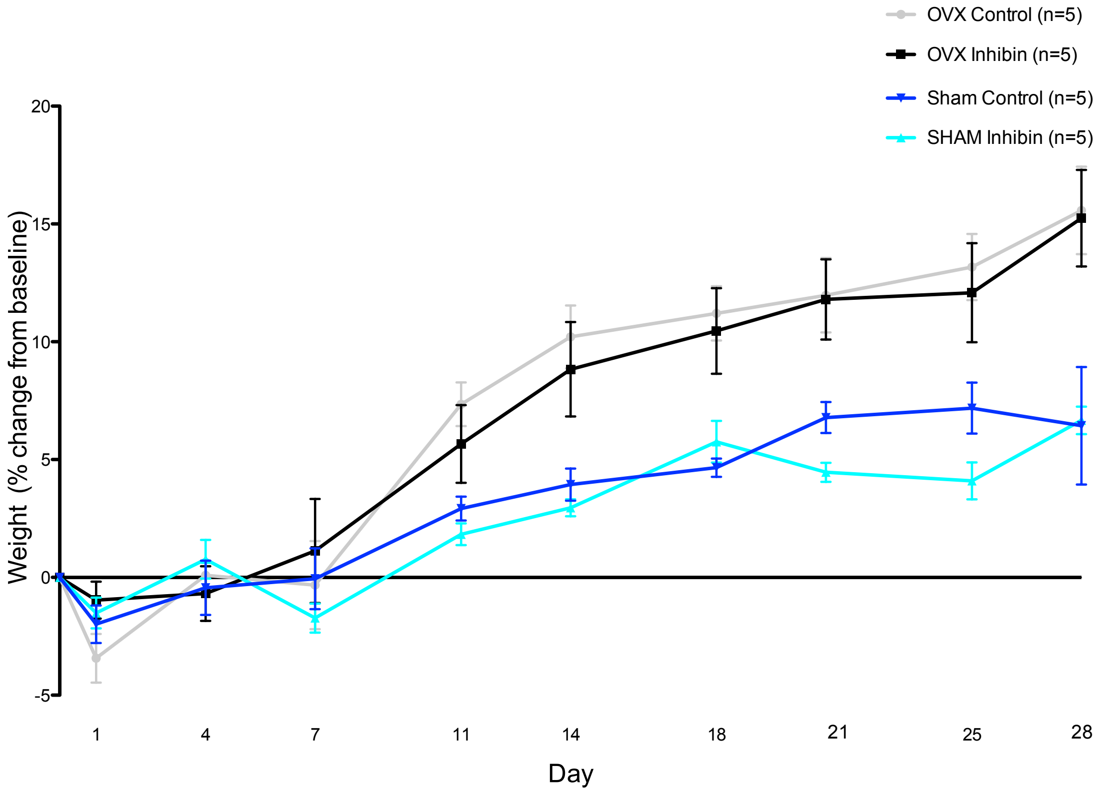


Figure 6.14. Percentage change in weight from baseline in mice treated with 120ng/day inhibin A after OVX or sham operation.

Mice were ovariectomised or sham-operated on day 0 followed by insertion of sub-cutaneous osmotic pump primed to deliver PBS or 120ng/day inhibin A. Baseline weight was measured after pump insertion and at regular timepoints over the 28-day course of the experiment. Data represents mean +SEM percentage change in weight over 28 days.

Serum inhibin A was significantly increased in OVX mice treated with inhibin A compared to OVX control (p=0.0079), Serum levels were also increased in sham animals treated with inhibin A compared to sham control, but this did not reach statistical significance (p=0.34) (Fig 6.15).

OVX induced bone loss compared to sham, and this was reversed by 120ng/day inhibin A (p=0.0079) (Fig 6.16A and 6.17A). Inhibin A also significantly increased bone volume compared to vehicle in sham-operated and OVX mice. Quantification of bone cell numbers on TRAP stained sections showed that OVX did not significantly alter osteoblast or osteoclast numbers compared to sham. In addition, inhibin A did not significantly alter the number of osteoblasts or osteoclasts compared to control in OVX or sham operated mice (Fig 6.16 B and 6.17 B+C).

Serum TRAP and P1NP were measured to determine the effects of OVX and inhibin A on bone cell activity. OVX did not significantly affect serum P1NP or TRAP compared to sham. Inhibin A did not significantly affect P1NP levels in OVX or sham operated animals, or alter TRAP levels in sham animals. However, administration of inhibin A in OVX mice significantly increased serum TRAP compared to OVX control mice (p=0.0079) (Fig 6.17 D+E).

6.5.4 Effects of inhibin A on activin and follistatin in the bone and liver in ovariectomised and sham-operated mice

Since inhibin A had a direct effect on breast cancer cell secretion of follistatin (section 6.5.1) which will change the activin:follistatin ratio in the paracrine tumour environment and potentially affect proliferation, the effects of inhibin A on the bone and liver microenvironment levels of activin and follistatin were evaluated. Calvaria and liver were collected from mice treated with 60ng/day inhibin A and 120ng/day inhibin A or PBS control for 28 days, calvaria were crushed in PBS and liver was homogenized in PBS to release soluble factors with storage of supernatant for processing to follistatin or activin ELISA (section 6.4.4) The calvaria from either sham operated or OVX mice treated with inhibin A 60ng/day showed no significant change in bone levels of either

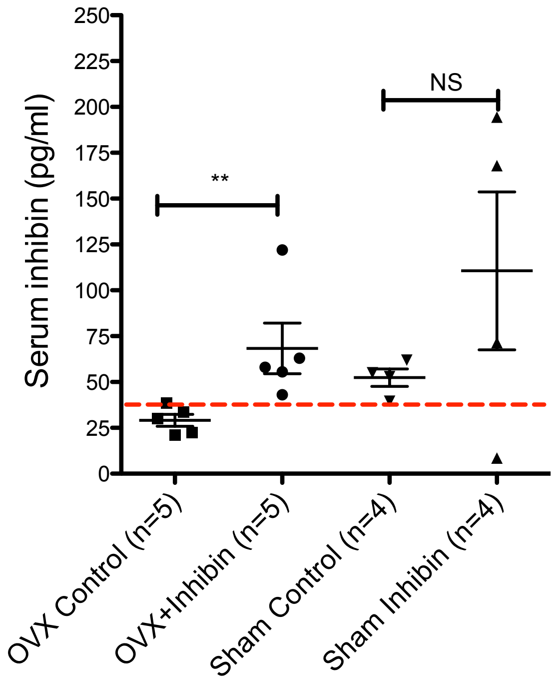


Figure 6.15. Serum levels of inhibin A in mice treated with and without 120ng/day inhibin A following OVX or sham operation.

Serum was collected at cull and precessed to human specific inhibin A ELISA. Data represents mean +SEM from **(A)** the overall population and (**B)** OVX and sham sub-groups of mice. Mann whitney test for significance. * p value <0.05, ** p value <0.01, NS not significant.
Red line represents lower limit of detection for assay

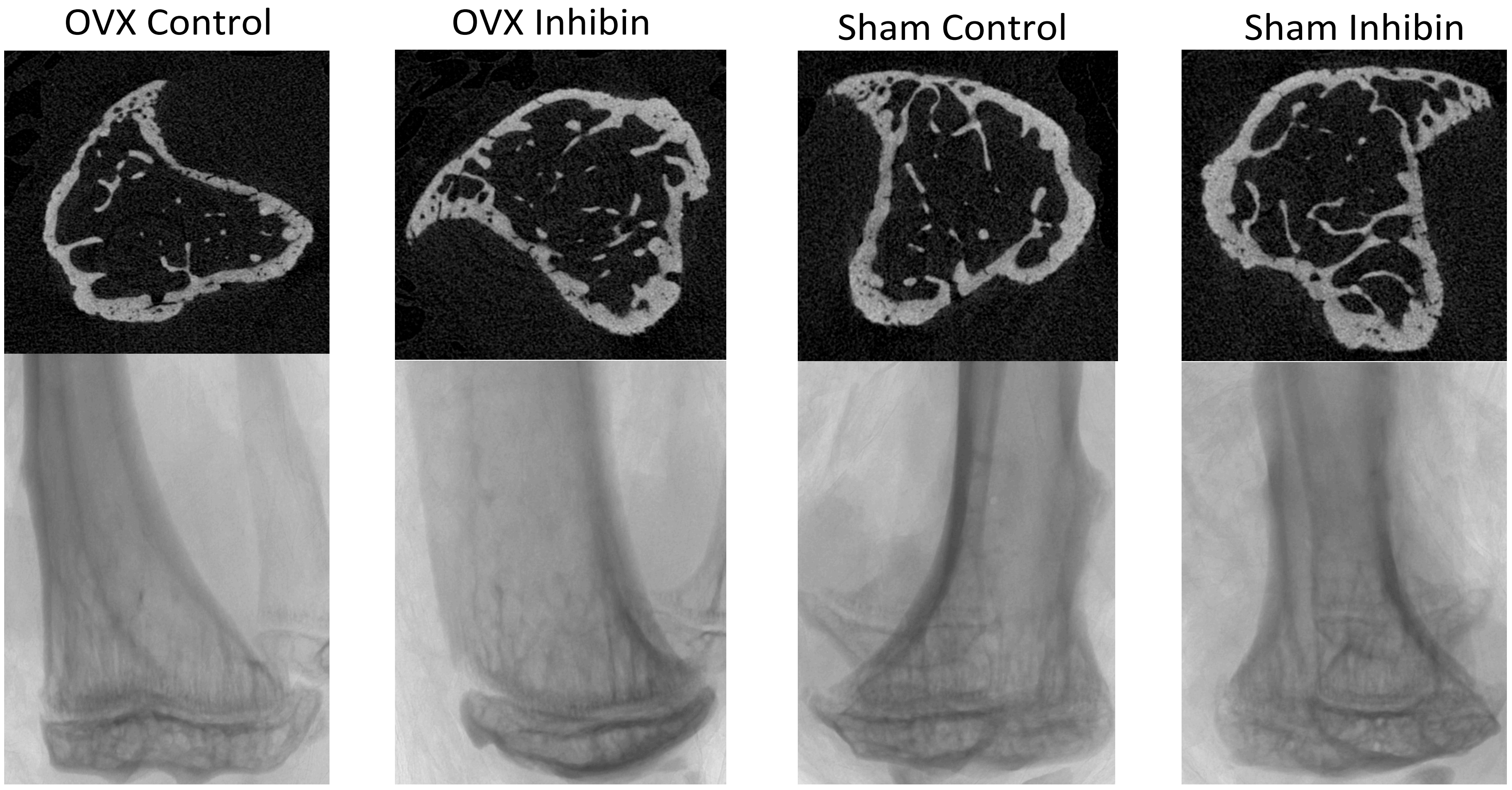


Figure 6.16a. Effect of 120ng/day inhibin on bone in OVX and sham mice – representative μCT images of proximal right tibia.

Representative μCT cross sectional images of the proximal right tibia (top) with associated radiographs (bottom) from OVX and sham mice treated with and without inhibin 120ng/day

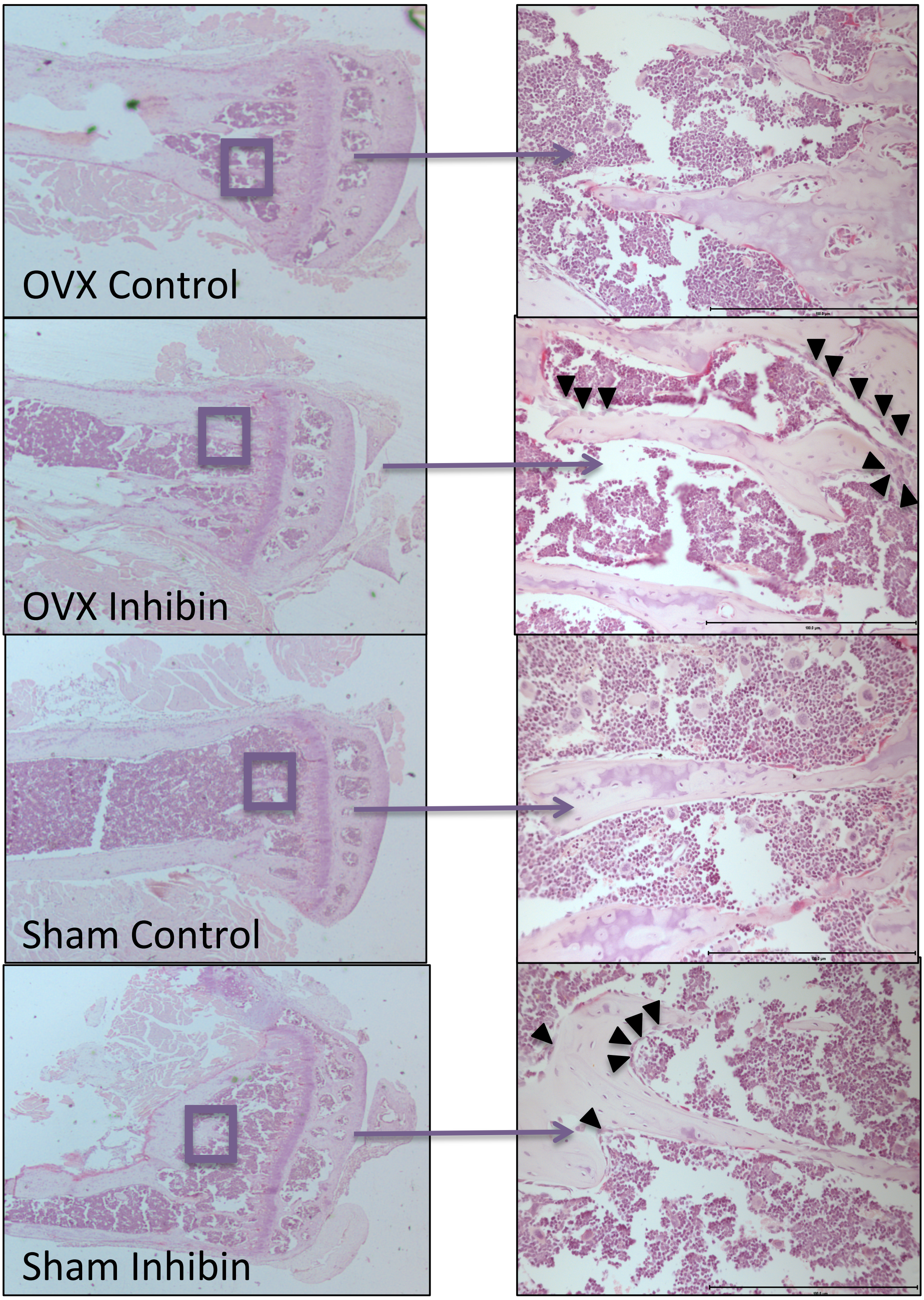


Figure 6.16b. Effect of 120ng/day inhibin A on osteoblasts and osteoclasts in OVX and sham mice- representative TRAP stained sections.

Arrowheads represent osteoblasts sitting on the surface of the bone. Purple box represents the area of magnification increased from x1.6 (left) to x20 (right). Scale bar represents 100μM.

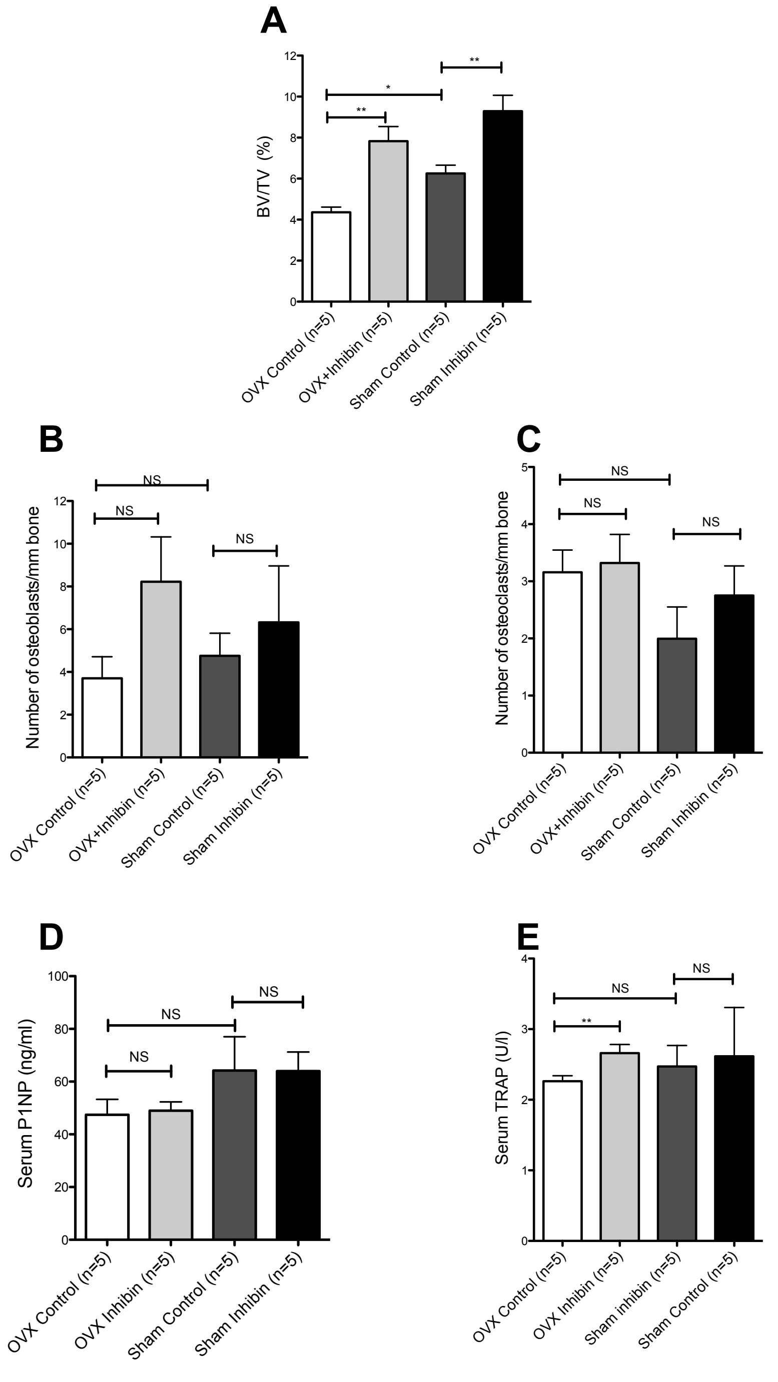


Figure 6.17. Effect of 120ng/day inhibin A on bone volume, osteoblast and osteoclast number and activity in animals following OVX or sham operation.

Mice were treated for 28 days with continuous sub-cutaneous infusion of 120ng/day inhibin A via ALZET pump, or PBS control, post OVX or sham operation.
A; BV/TV in OVX and sham animals assessed using μCT imaging. **B+C;** Osteoblast and osteoclast number per mm bone quantified on TRAP stained histological sections. **D+E** Serum P1NP as a marker of osteoblast activity and serum TRAP as a marker of osteoclast activity. All data represent mean +SEM, Mann Whitney U test for significance, ** p value <0.01, * p value <0.05, NS not significant.

activin or follistatin compared to control (Fig 6.18 A +B). Calvaria from OVX mice treated with 120ng/day inhibin A showed a significant decrease in activin levels compared to OVX control (p=0.01) (Fig 6.18 A+C). This effect was not seen in the sham-operated animals. There was no significant change in follistatin levels with inhibin A from calvaria in either OVX or sham animals (Fig 6.18 B+D).Inhibin A did not alter activin levels in the liver of either sham or OVX animals. The levels of liver follistatin were below the level of detection for the ELISA in all groups.

6.5.5 Effects of zoledronic acid on the bone microenvironment in ovariectomised and sham-operated mice. Are levels of follistatin and activin altered?

In order to evaluate the effects of zoledronic acid on the bone microenvironment zoledronic acid treatment was commenced on day 1 of treatment protocol, and mice underwent OVX or sham operation on day 2. Mice were maintained for 28 days and treated weekly with either 100µg/kg of zoledronic acid or saline control via intra peritoneal injection. Weights were recorded over the duration of the experiment and showed a decrease from baseline for the first 5 days, with recovery to baseline and above thereafter (Fig 6.19).

As expected OVX induced bone loss compared to sham and weekly administration of zoledronic acid significantly increased bone volume compared to control in both OVX and sham operated mice compared to saline control (OVX p= 0.016, sham p= 0.0079) (Fig 6.20A and 6.21A). Quantification of bone cell numbers showed that OVX did not alter osteoblast or osteoclast number compared to sham and zoledronic acid did not significantly alter the number of osteoclasts/mm bone compared to control in the OVX/sham animals. However, there was a significant decrease in osteoblasts/mm bone in zoledronic acid treated mice compared to control in both the OVX and sham animals (OVX p=0.007, sham p=0.009) (Fig 6.20 B and 6.21B+C).

Serum TRAP and P1NP levels were measured to assess effects on osteoclast and osteoblast activity. OVX did not alter P1NP or TRAP levels compared to sham. Zoledronic acid significantly decreased TRAP in both OVX and sham animals (OVX p=0.0079, sham p=0.016).

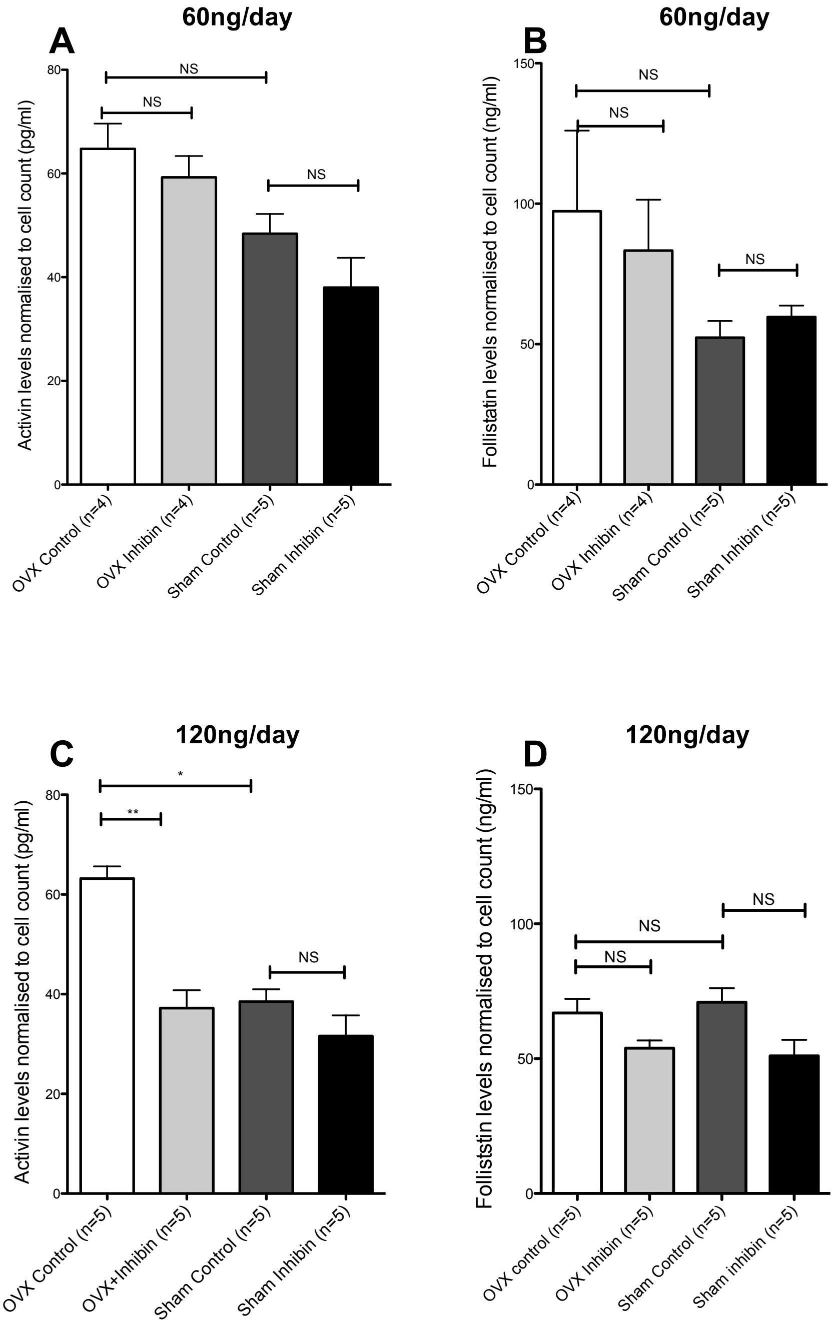
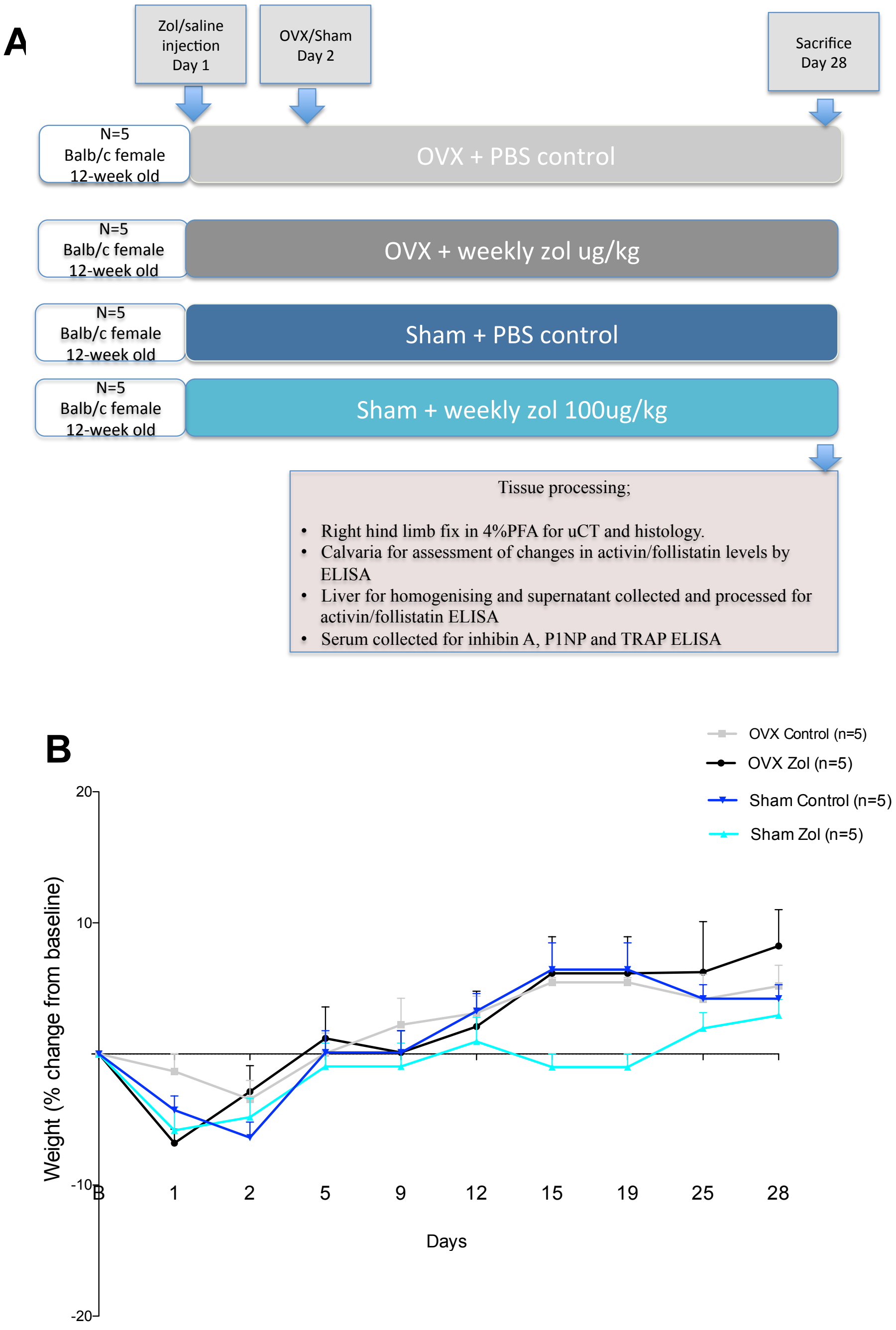


Figure 6.18 Effect of inhibin A on bone levels of activin and follistatin in OVX and sham operated mice.

Mice were treated for 28 days with either PBS control, 60ng/day or 120ng/day of inhibin A post OVX or sham operation. **A+B**; Calvaria levels of activin and follistatin in mice treated with 60ng/day inhibin or PBS control. **C+D**; Clavaria levels of activin and follistatin in mice treated with 120ng/day inhibin or PBS control. All data represent mean+SEM, Mann Whitney test for significance, * p value <0.05, *** p value <0.001, NS not significant.



Tissue processing;

- Right hind limb fix in 4%PFA for uCT and histology.
- Calvaria for assessment of changes in activin/follistatin levels by ELISA
- Liver for homogenising and supernatant collected and processed for activin/follistatin ELISA
- Serum collected for inhibin A, P1NP and TRAP ELISA

Figure 6.19. **Experimental outline to determine the effects of zoledronic acid (zol) on the bone microenvironment in OVX and sham operated mice.**

A; Weekly IP injections commenced on day 1 with 100µg/kg of zoledronic acid. On day 2 they underwent OVX (n=10) or sham (n=10) operation. Mice were maintained for 28 days with tissue collected for downstream analyses as outlined. **B;** Serial measurements of weight over duration of experiment, data represent mean +SEM percentage change from baseline weight over 28 days.

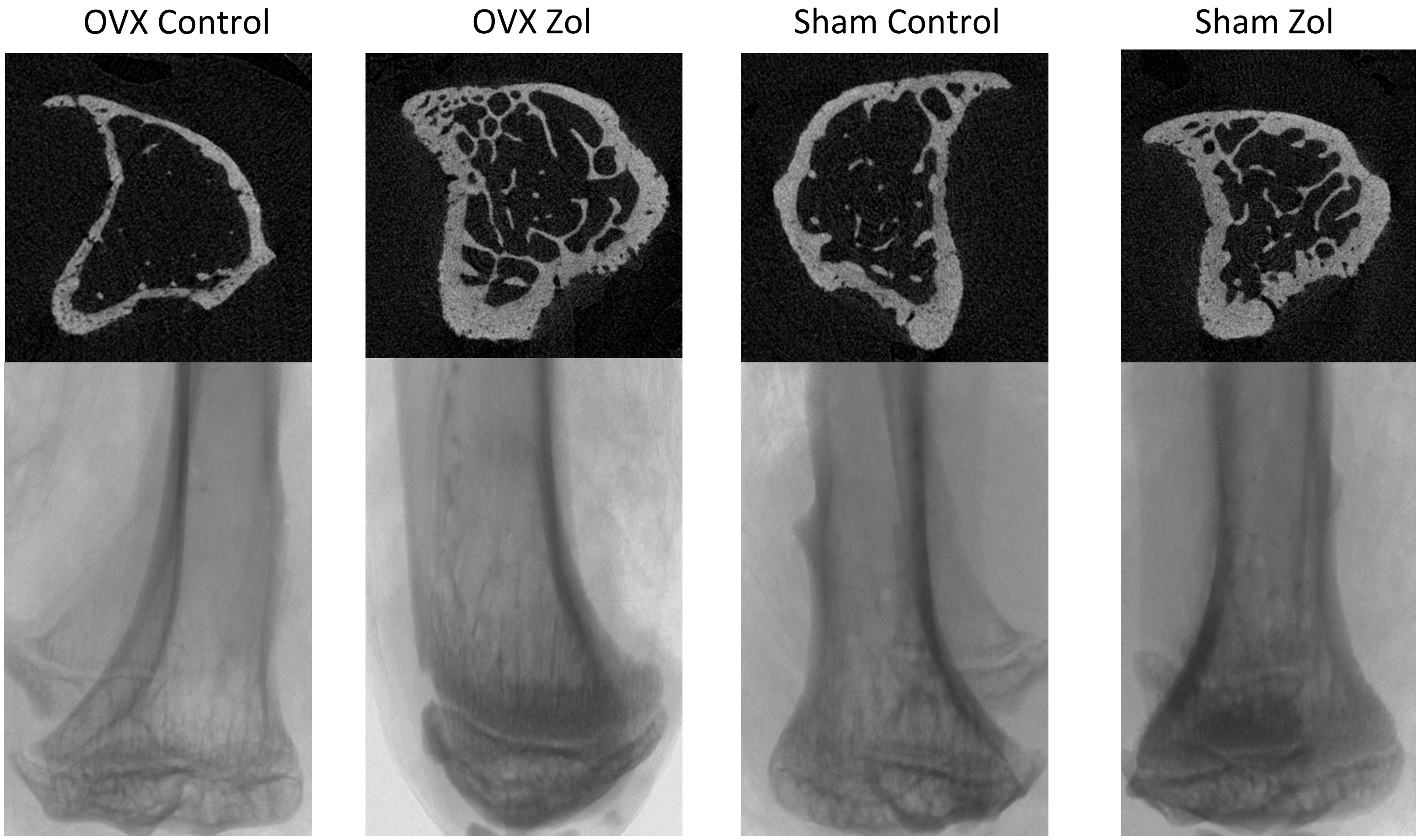


Figure 6.20a. Effect of zoledronic acid on bone in OVX and sham mice- representative μCT images of proximal right tibia.

Representative μCT cross sectional images of the proximal right tibia (top) with associated radiographs (bottom) from OVX and sham mice treated with and without zoledronic acid.

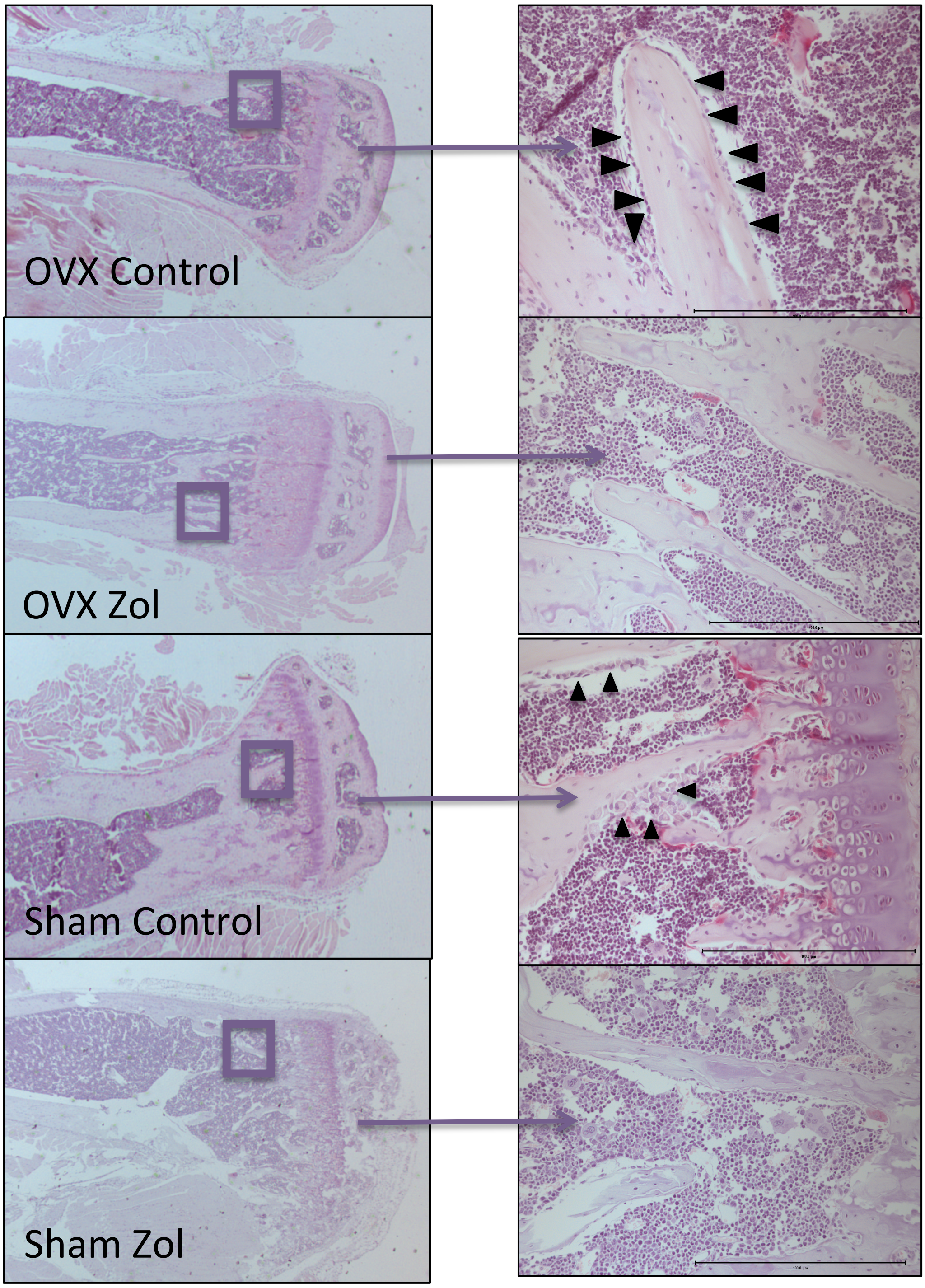


Figure 6.20b Effect of zoledronic acid on osteoblast and osteoclast numbers in bone in OVX and sham operated mice- representative TRAP stained sections.

Black arrowheads represent osteoblasts. Purple box represents section magnified from x1.6 (left) to x20 (right). Scale bar represents 100µm.

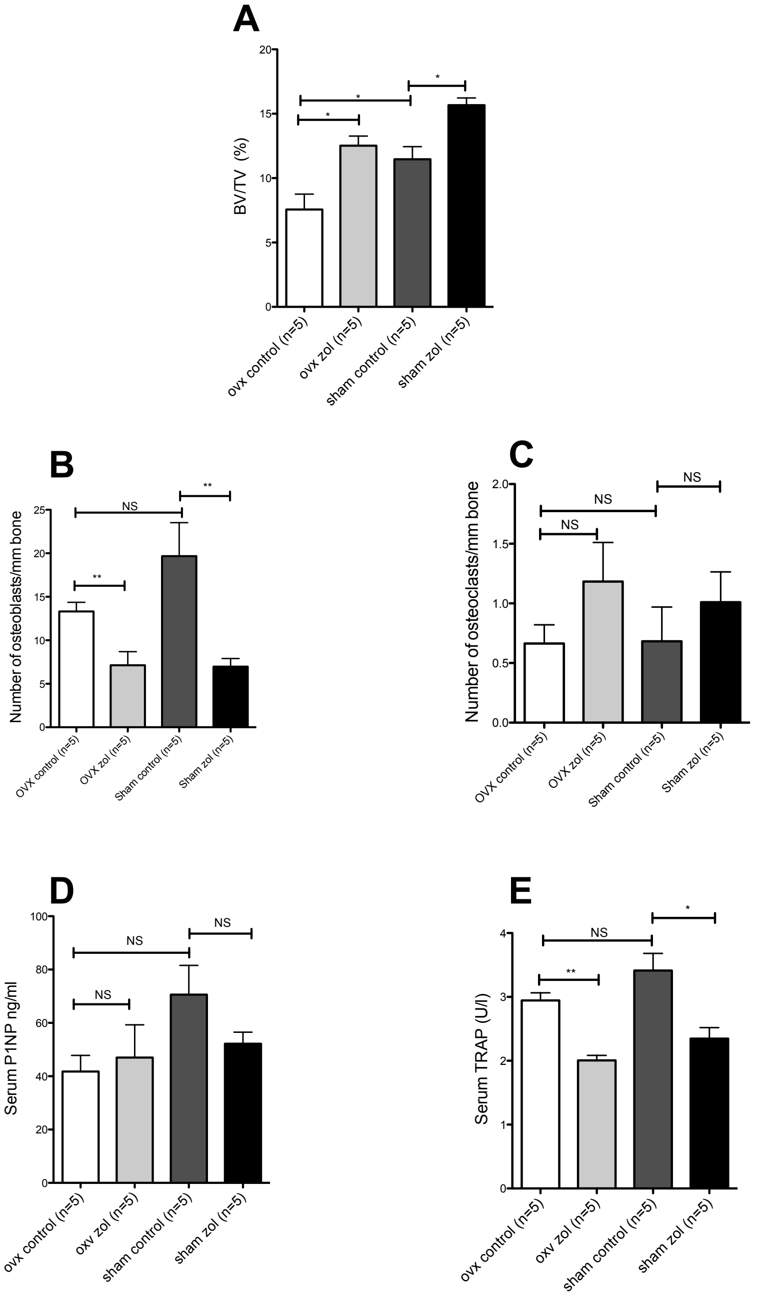


Figure 6.21. Effect of zoledronic acid (zol) on bone volume, osteoblast and osteoclast number and activity in animals following OVX or sham operation.

Mice were treated for 28 days with weekly IP injection of 100μg/kg of zoledronic acid or PBS control, post OVX or sham operation. **A**; BV/TV in OVX and sham animals assessed using μCT imaging. **B+C;** Osteoblast and osteoclast number per mm bone quantified on TRAP stained histological sections. **D+E** Serum P1NP as a marker of osteoblast activity and serum TRAP as a marker of osteoclast activity. All data represent mean +SEM, Mann Whitney U test for significance, * p value <0.05,** p value <0.01, NS not significant.

There was no significant change in P1NP in either OVX or sham animals with zoledronic acid (Fig 6.21 D +E). Zoledronic acid did not significantly alter activin levels in the calvaria in OVX or sham animals, However, follistatin was significantly decreased by zoledronic acid in the OVX animals compared to control (p=0.036), this effect was not seen in sham animals (Fig 6.22). Liver activin levels were unaffected by zoledronic acid in both groups.

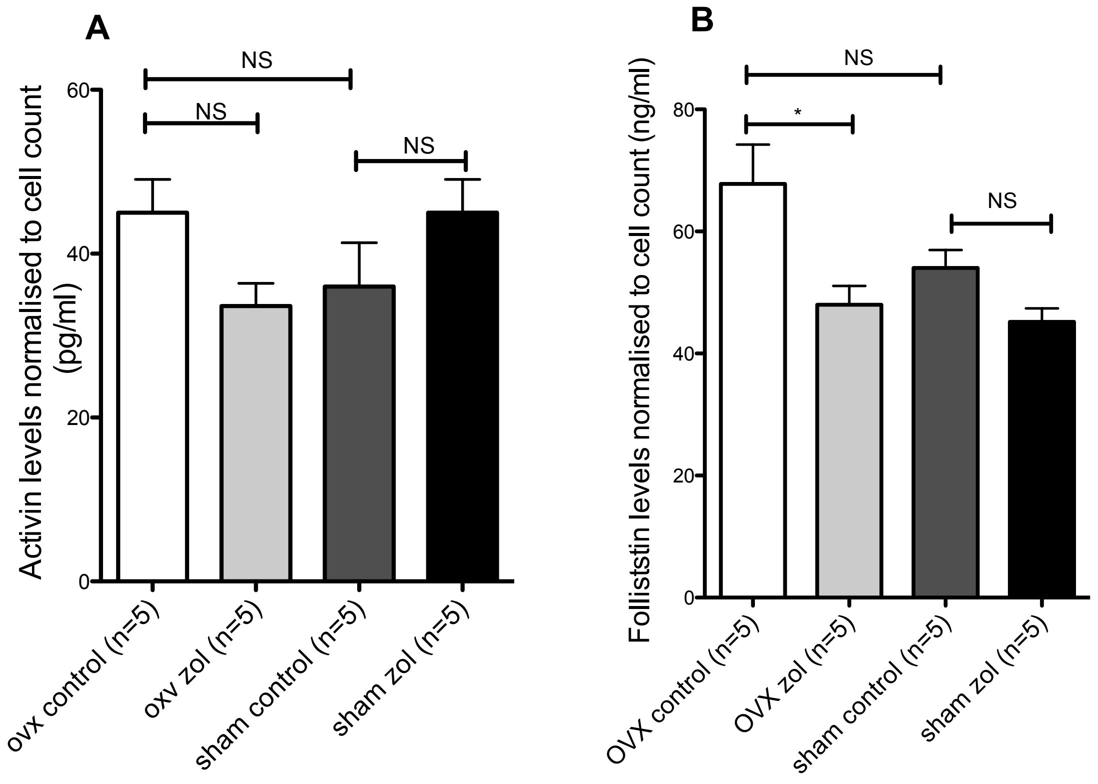


Figure 6.22 Effect of zoledronic acid on bone levels of activin and follistatin in OVX and sham operated mice.

Mice were treated for 28 days with weekly IP injection of 100μg/kg zoledronic acid or PBS control, post OVX or sham operation. At the end of the experiment calvaria were collected and crushed in a fixed volume of PBS to release soluble factors. PBS + bone was filtered and centrifuged and supernatant was removed for processing to activin and follistatin ELISA. **A**; Calvaria levels of activin in mice treated with zoledronic acid or PBS control. **B**; Calvaria levels of follistatin in mice treated with zoledronic acid or PBS control. All data represent mean+SEM, Mann Whitney test for significance, *p value <0.05, NS not significant.

6.6 Discussion

The data presented in this chapter suggest that inhibin A can prevent the decrease in secreted follistatin induced by zoledronic acid in ER-ve breast cancer cells *in vitro*. *In vivo* the recombinant inhibin A protein from NIBSC appears to be biologically active, and at the highest dose used (120ng/day for 28 days) increases bone volume in both OVX and sham animals. It also decreases activin levels in calvaria in OVX animals. Zoledronic acid, as expected, increases bone volume in both OVX and sham animals, via a reduction in activity of osteoclasts, with a concurrent decrease in osteoblast numbers. It also decreases follistatin levels in calvaria in OVX animals. This would indicate that in an OVX animal, with low inhibin A, bone volume will be reduced but bone (calvaria) activin levels are high; if zoledronic acid is present then bone volume will increase, and bone follistatin levels will fall, further increasing bioavailability of activin in bone. In an animal with high inhibin A levels (i.e. OVX+inhibin A), bone volume will be high but bone activin levels are low; if zoledronic acid is present then bone volume will be further increased but no change in bone follistatin will occur. This may potentially create two different bone microenvironments, which could differentially affect tumour cell growth and progression (Fig 6.24).

However, there are limitations to interpretation of this data. As previously discussed the direct effect of inhibin A on breast cancers is not clear. The data presented in this chapter suggests that inhibin A counteracts the effects of zoledronic acid on follistatin secretion from MDA-MB-231 cells *in vitro* by increasing follistatin secretion from the cells. Although there are no previously published studies, to my knowledge, of the effect of inhibin A on follistatin secretion from breast cancer cells, there has been published data from both *ex vivo* and *in vivo* pituitary cultures suggesting inhibin A can affect follistatin expression. Rat anterior pituitary cells treated with inhibin A for 24 hours showed a significant decrease in the expression of follistatin mRNA (34% of control) (Bilezikjian, Corrigan *et al*. 1996). This was recently confirmed in an *in vivo* experiment using ovariectomised 3 month old female rats, showing a significant increase in mRNA levels of follistatin in the pituitary at day 28 post OVX compared to 1 day post OVX (Popovics, Rekasi *et al*. 2011). These data suggest high inhibin A levels suppress follistatin expression in pituitary cells.

	Low inhibin	High inhibin
Bone volume	↓	↑
Osteoblast number	↔	↔
P1NP	↔	↔
Osteoclast number	↔	↔
TRAP	↓	↑
Bone (calvaria) activin	↑	↓
Bone (calvaria) follistatin	↔	↔

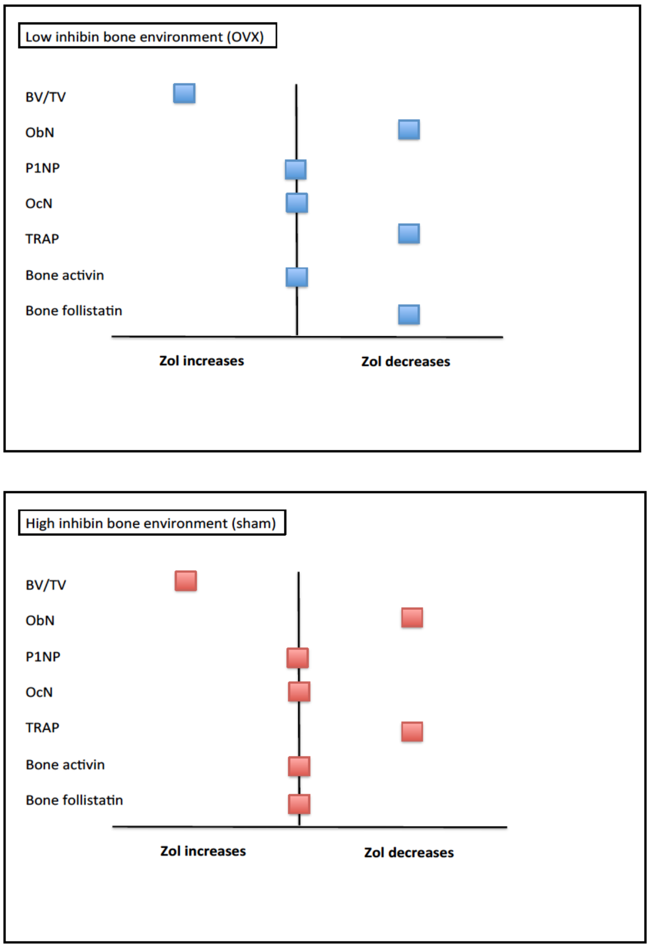


Figure 6.24 Summary of changes in the bone microenvironment with both inhibin A and zoledronic acid.

In a low inhibin bone environment activin levels are high. Addition of zoledronic acid to a low inhibin environment (OVX) decreases bone follistatin levels thus further increasing bioavailability of activin. In a high inhibin bone environment activin levels are low and zoledronic acid does not affect follistatin.

This is in contrast to my findings in MDA-MB-231 cells, however, it does demonstrate that inhibin A can directly affect follistatin secretion from cells, but the direction of affect is likely to be cell type dependent.

The serum levels attained with the use of the ALZET osmotic pumps were lower than expected considering the high doses added to the pumps, with only the 120ng/day delivery system significantly increasing serum inhibin A in OVX animals. The serum follicle stimulating hormone (FSH) were not consistent with published literature. Inhibin A is a negative regulator of FSH, therefore as serum inhibin A levels increase, FSH levels would be expected to fall. Carl *et al* used a rat model of ovariectomy to demonstrate the effects of a continuous infusion of porcine follicular fluid (containing high doses of inhibin A, but charcoal stripped of oestrogen) on serum FSH levels. FSH was suppressed in treated animals compared to control up to a 20 day time-point, However, the suppression of FSH was lost at the 25 day time-point in treated animals and FSH fell to comparable levels with control rats (Carl L 1984). These data suggest that a continuous exposure to high doses of inhibin A over 25 days will negate the suppressive effect of inhibin A on FSH secretion. This may explain why FSH was not significantly altered compared to control when mice were treated with continuous inhibin A for 28 days in the current study.

Although FSH levels did not convincingly demonstrate biological activity of the recombinant inhibin A used, the effects in bone were more supportive of biological activity. The effect on the bone microenvironment can be compared to the published *in vivo* studies using either transgenic mice overexpressing inhibin A or mice treated with an ActRIIA inhibitor, which will have the same molecular effect as recombinant inhibin A. Perrien *et al* used transgenic mice induced to secrete large quantites of inhibin A from liver in response to mifepristone (MFP). Serum levels of inhibin A in the transgenic mice were 400-800pg/ml; they found inhibin A increased bone density in intact females and in sham/orchidectomy males. They found no significant change in osteoblasts or osteoclasts with inhibin A, but a significant increase in serum osteocalcin, a marker of osteoblast activity (Perrien, Akel *et al.* 2007). My data recorded lower levels of serum inhibin A (maximum 110pg/ml sham+inhibin A), but a consistent increase in bone density in both OVX and sham females, however, there was no significant increase in either osteoblast number or P1NP, a marker of osteoblast activity. In fact the opposite was demonstrated in OVX animals with an increase in serum TRAP, a marker of osteoclast activity. This may reflect the different doses of serum inhibin A achieved in the two experiments and the different markers of bone formation evaluated.

Rissanen *et al* performed a longitudinal study in OVX and sham rats to evaluate the correlation of serum P1NP with other markers of bone formation; osteocalcin and bone resorption; CTX, and trabecular bone assessment. P1NP increased significantly in the first 2 weeks after OVX but returned to baseline at 8 weeks, and the changes in P1NP correlated significantly with changes in serum osteocalcin and CTX at the 2-week time-point only, but with trabecular bone parameters at 8 weeks (Rissanen, Suominen *et al.* 2008). This suggests that changes in P1NP occur early after an intervention such as OVX, and that these levels subsequently normalize, however, changes in bone trabecular measurements occur later when serum markers have normalized. Therefore the lower serum level of inhibin A in my experiments compared to the transgenic model, may have induced changes in osteoblast activity that had normalised by the 4-week time-point. The expected coupling of bone formation to bone resorption could then have caused a later effect on osteoclast activity, which was detectable at the 4-week time-point.

This theory is supported by the work of Peasall *et al* who injected 12-week old female intact mice with an activin receptor inhibitor, ActRIIA-mFC (10mg/kg), and evaluated the effect on bone in a timecourse experiment at 2, 4, 6 and 12-weeks. They found the ActRIIA-mFC caused a significant increase in serum osteocalcin at 2 and 4 weeks, but also that serum TRAP significantly increased at week 4 in the treatment group. The results indicate that the coupling of bone formation to bone resorption cause a delayed detectable effect on osteoclast activity after the earlier changes in osteoblast activity. Osteoblast and osteoclast numbers were not reported in this paper (Pearsall, Canalis *et al.* 2008).

Chantry *et al* performed a similar experiment in 8-week old intact female C57/BL/6 mice treated for 6 weeks with increasing doses of ActRIIA-mFC. There was a significant increase in osteoblast numbers in treated vs control mice which was reflected by a dose dependent increase in bone volume (Chantry, Heath *et al.* 2010). These data therefore suggest that blocking the ActRIIA receptor with either inhibin A or ActRIIA-mFC causes an early increase in osteoblast activity detectable at 2-4 weeks, with a coupled delayed effect on osteoclasts detectable from 4 weeks onwards, and a net effect on osteoblast numbers that is detectable at a later time-point of 6 weeks. These data therefore support my findings, adding confidence that the recombinant inhibin A protein from NIBSC had biologically relevant effects in bone.

Several *in vivo* studies have demonstrated that bisphosphonates reduce osteoclast numbers in both the absence (Kuroshima, Go *et al.* 2012) (Hughes, Wright *et al.* 1995; Ito, Amizuka *et al.* 1999) and presence of tumour cells (Walker, Medhurst *et al.* 2002; Ottewell, Woodward *et al.* 2009) (Martin, Werbeck *et al.* 2010). However, the effect of zoledronic acid on osteoblasts is less well documented. *In vitro* studies have suggested zoledronic acid inhibits osteoblast activity (Naidu, Dechow *et al.* 2008; Basso, Silveira Turrioni *et al.* 2013), and in an *in vivo* study of 6-week old balb/c nude mice treated with a single dose of 100μg/kg zoledronic acid, with bone assessment at day 1, 3, 5 and 10 days post treatment, showed a significant reduction in osteoblast number and serum P1NP as early as day 3 (Haider M 2013). Brown *et al* evaluated the treatment effects of doxorubicin or zoledronic acid or combination therapy on MDA-MB-231 tumour growth in bone after intracardiac injection in 6-week old female balb/c nude mice. In the control, non-tumour bearing animals, a single IP injection of 100μg/kg of zoledronic acid significantly decreased osteoblast number at day 15 and 23 after injection, with a concurrent decrease in P1NP at day 15 that was lost at day 23 (Brown HK 2012). My data show that zoledronic acid does not significantly alter osteoclast numbers after 4 weeks of weekly treatment but does impair their activity as evidenced by a significant decrease in serum TRAP in OVX and sham animals. There was a significant reduction in osteoblast numbers in both sham and OVX animals treated with zoledronic acid; whether this is a direct effect of zoledronic acid on the osteoblast, or a delayed effect due to uncoupling of bone turnover due to an earlier effect on osteoclasts is not clear.

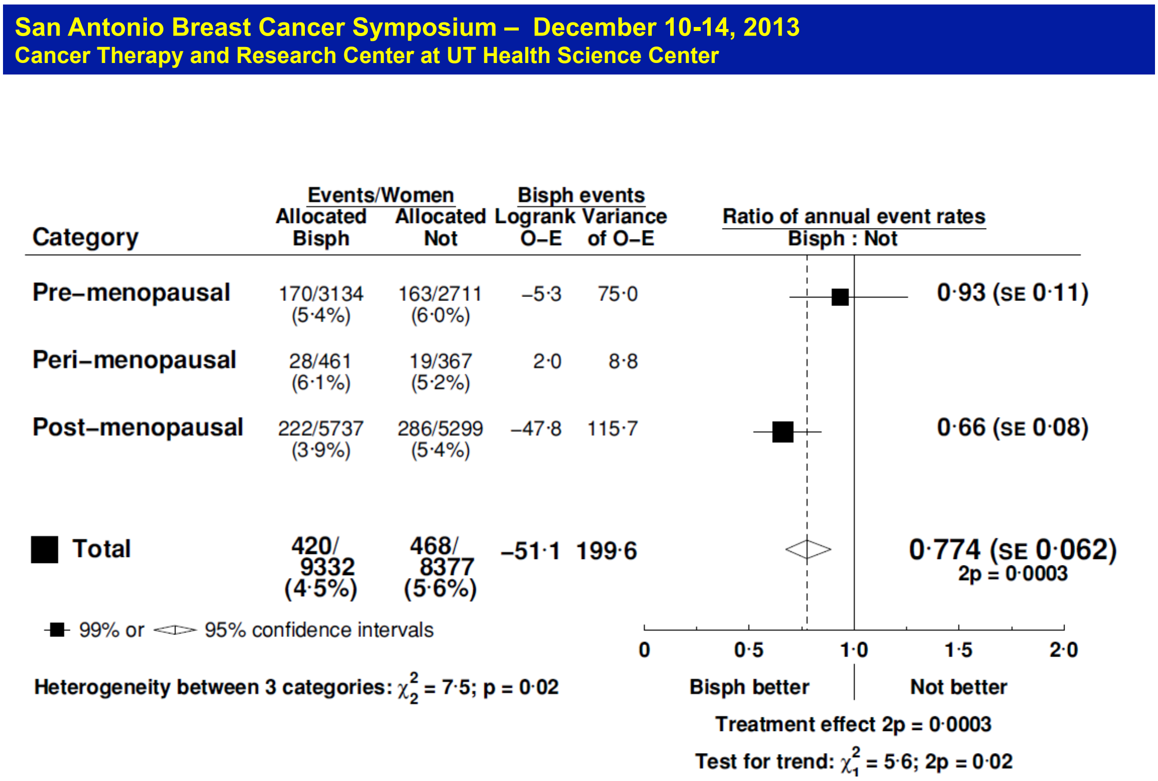
In summary inhibin A increased bone volume and decreased bone (clavaria) levels of activin. Zoledronic acid increased bone volume and decreased osteoblast numbers and osteoclast activity in sham and OVX animals. Moreover, zoledronic acid decreased bone (calvaria) levels of follistatin only in the absence of inhibin A (OVX animals). These data suggest that inhibin A has biological effects in bone, and could potentially influence the effect of zoledronic acid on bone levels of the paracrine tumour suppressor activin and tumour promoter follistatin. These data are hypothesis generating, and a combined *in* *vivo* experiment with both inhibin A and zoledronic acid would be needed to identify if the combination treatment would have the suggested effect on bone activin and follistatin levels, and subsequent effect on tumour cells within the bone microenvironment

Chapter 7. General discussion.

The differential anti-tumour effect of adjuvant zoledronic acid, according to the menopausal status of breast cancer patients, was a novel finding in the AZURE trial. Zoledronic acid significantly decreased bone and non-bone distant recurrences in women who were clinically >5 years postmenopausal, however, non-bone recurrences were increased in all other women (Coleman, Marshall *et al.* 2011). It was therefore considered a priority to reliably categorised menopausal status to prevent administration of zoledronic acid to patients who may derive harm from the drug. Moreover, the molecular mechanisms driving this hormone:zoledronic acid interaction were unknown, and whether it was due to single or multiple reproductive hormones.

The evaluation of pretreatment FSH, oestradiol and inhibin A from 806 patients in the AZURE trial attempted to assess if selection of patients for adjuvant zoledronic acid on the basis of a biochemical postmenopausal status was possible, rather than relying on patient reported recall in clinic which can be prone to error (Colditz, Stampfer *et al.* 1987). The concordance of clinically reported menopausal status and biochemical menopausal status was not 100%. This was likely due to older menstruating patients appearing biochemically postmenopausal due to anovulatory menstrual cycles, and patients many years postmenopausal appearing biochemically non-postmenopausal due to oestradiol levels that were above the specified postmenopausal cut point (<50pmol/l). However, notwithstanding this disparity, the IDFS outcomes with zoledronic acid in patients defined as postmenopausal solely using a pretreatment biochemical evaluation of FSH, oestradiol and inhibin A, continued to select a population that derived improvements in IDFS both in bone and outside of bone. This suggested that a baseline reproductive hormones have a role in selection of patients for adjuvant zoledronic acid, but since clinical and biochemical menopausal status is not always concordant, they should both be evaluated and interpreted with respect to one another. In patients who's clinical menopausal status was unknown or they were within a dynamic period of change in hormone levels i.e. <5 years since menopause, the AZURE results indicated biochemical evaluation of reproductive hormones may assist in selecting these patients for adjuvant zoledronic acid, but they are likely to need serial evaluations rather than a single pretreatment levels due to fluctuations in hormone levels over time (Burger 2011). These data were reassuring in that a biochemical pretreatment evaluation of reproductive hormones may be able to prevent zoledronic acid being administered to patients with an active HPG axis who may potentially experience a detrimental effect from the drug.

However, the concern over harm and increased non-bone recurrences in non-postmenopausal women treated with zoledronic acid has not been confirmed in large meta-analyses of adjuvant bisphosphonates recently reported. A meta-analysis of adjuvant zoledronic acid trials evaluated overall survival from 5 randomised trials (n=6414) (Valachis, Polyzos *et al*. 2013). Zoledronic acid therapy commenced at the time of diagnosis resulted in a significant improvement in overall survival (HR 0.81; 95% CI 0.70-0.94 p value 0.007). The trials included in this analysis were in either a mixed menopausal population AZURE (n=3370)(Coleman, Marshall *et al*. 2011) and Aft *et al* (n=119) (Aft, Naughton *et al*. 2010), a postmenopausal population ZO-FAST (n=1065) (Eidtmann, de Boer *et al*. 2010) and Leal *et al* (n=68)(Leal, Tevaarwerk *et al*. 2010), and a premenopausal population with chemically induced menopause ABCSG-12 (n=1803)(Gnant, Mlineritsch *et al*. 2011). The authors stressed that the beneficial effect of zoledronic acid in low oestrogen environments may be even greater than that demonstrated in these mixed menopausal population trials. Reassuringly the potential 'harm' seen in the AZURE trial in non-postmenopausal women was not confirmed in this meta-analysis. The largest meta-analysis of adjuvant bisphosphonate trials was presented in December 2013 at the San Antonio Breast Cancer Symposium and included data from ~23,000 pre- and postmenopausal patients treated with adjuvant bisphosphonates, including both non-nitrogen, and nitrogen-containing bisphosphonates. Primary outcome measures were time to recurrence, time to distant recurrence including bone and non-bone and breast cancer mortality. Bisphosphonates significantly decreased bone recurrence in the overall population compared to control (6.9% vs. 8.4% p=0.0009) but had no effect on non-bone recurrence. The reduction in bone recurrence was driven by postmenopausal patients (natural or chemically induced) (Fig 7.1). In addition there was a non-significant trend to a reduction in non-bone recurrence in postmenopausal women (9%, 95%CI 5%-19%), and 10-year breast cancer mortality was significantly improved in these women with addition of bisphosphonates to standard adjuvant therapy (15.2% vs. 18.3% p=0.004) (Coleman RE 2013). The clinical definition of postmenopausal used in the meta-analysis did not differentiate between women 1-5 years postmenopausal and >5 years postmenopausal as in the AZURE trial. The standard World Health Organization clinical definition of natural menopause is; 'the permanent cessation of menstruation determined retrospectively after 12 months of amenorrhoea without any other pathological or physiological cause' (WHO 1996). In the meta-analysis, the beneficial effect of zoledronic acid on bone recurrence in women who were clinically



San Antonio Breast Cancer Symposium – December 10-14, 2013
Cancer Therapy and Research Center at UT Health Science Center

Category	Events/Women Allocated Bisph	Events/Women Allocated Not	Bisph events Logrank O−E	Bisph events Variance of O−E	Ratio of annual event rates Bisph : Not
Pre−menopausal	170/3134 (5·4%)	163/2711 (6·0%)	−5·3	75·0	0·93 (SE 0·11)
Peri−menopausal	28/461 (6·1%)	19/367 (5·2%)	2·0	8·8	
Post−menopausal	222/5737 (3·9%)	286/5299 (5·4%)	−47·8	115·7	0·66 (SE 0·08)
■ **Total**	**420/9332 (4·5%)**	**468/8377 (5·6%)**	**−51·1**	**199·6**	**0·774 (SE 0·062) 2p = 0·0003**

■ 99% or ◇ 95% confidence intervals

Heterogeneity between 3 categories: χ^2_2 = 7·5; p = 0·02

Treatment effect 2p = 0·0003

Test for trend: χ^2_1 = 5·6; 2p = 0·02

Figure reproduced with permission from RE Coleman. Effects of bisphosphonate treatment on recurrence and cause-specific mortality in women with early breast cancer: A meta-analysis of individual patient data from randomized trials. Presented at San Antonio Breast Cancer Symposium, Dec 2013. Abst; S4:07

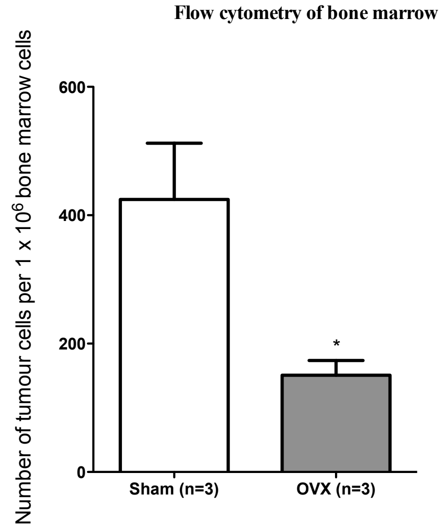
Figure 7.1 Bone recurrences according to menopausal status from a large meta-analysis of adjuvant bisphosphonate trials.

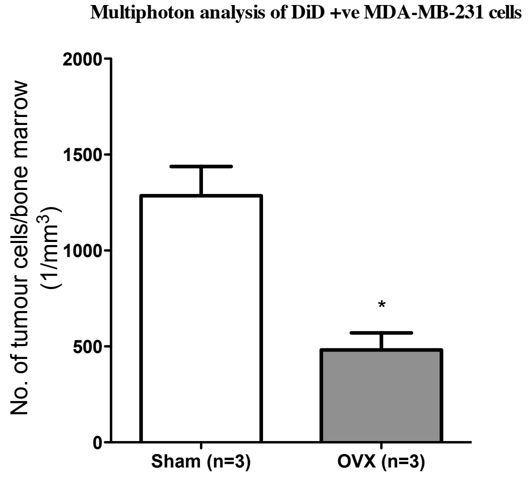
Bone recurrence was significantly reduced in the overall meta-analysis population (n= 22982). This effect was driven by the postmenopausal population, with a significant difference in the benefit gained from bisphosphonates in premenopausal patients (HR 0.93, SE 0.11) compared to postmenopausal patients (HR 0.66, SE 0.08) p value 0.02.

postmenopausal (natural or chemically induced) was confirmed, despite some of these women being within 1-5 years of menopause. However, no significant anti-tumour effect of zoledronic acid on recurrences outside of bone was shown, in contrast to the AZURE patients who were >5 years postmenopausal. These data suggest that as women are chronologically further away from menopause, the effects of adjuvant bisphosphonates on disease recurrence may be greater, with beneficial effects both in bone and outside of bone. Moreover, the detrimental effect of zoledronic acid on disease recurrences outside of the bone in patients <5 years postmenopausal in the AZURE trial, was not confirmed in the large meta-analysis. It therefore conceivable that a clinical definition of postmenopausal based on the widely accepted WHO definition (12 months of amenorrhoea) will be utilised in selecting patients for adjuvant zoledronic acid. Pretreatment biochemical classification of menopausal status may therefore be of more importance in patients whose clinical status is unknown, and the data presented in chapter 3 (Fig 3.4) has shown this may be possible using FSH, oestradiol and inhibin A.

As discussed earlier, the molecular mechanisms driving this hormone:zoledronic acid interaction were also unknown, including whether this was due to single or multiple reproductive hormones. At a molecular level it was undetermined if this was a direct effect on tumour cells or an indirect effect by differential modification of the bone microenvironment. The AZURE data presented in chapter 3 showed that high versus low levels of FSH/oestradiol/inhibin A were not predictive of a response to zoledronic acid on disease recurrence in or outside of bone, However, there was a trend to benefit from zoledronic acid in patients with hormone levels in the postmenopausal range (high FSH, low oestradiol and inhibin)(table 3.4). This indicated that no single hormone was modifying the effects of zoledronic acid, and that the overall activity of the hypothalamic pituitary axis is more important in determining response to the drug. The patient cohorts recruited to the clinical trials that showed a beneficial effect of zoledronic acid in reducing disease recurrence, included both naturally postmenopausal women (Coleman, Marshall *et al.* 2011; Coleman, de Boer *et al.* 2013) and chemically induced postmenopausal women (Gnant, Mlineritsch *et al.* 2011). Both naturally and chemically induced postmenopausal women will have low oestradiol and inhibin A but they will have different FSH levels, being high in the former and low in the later. This suggests that ovarian hormones, rather than pituitary hormones are more important in modifying the anti-tumour effects of zoledronic acid.

Although ovarian hormones may be driving the anti-tumour interaction with zoledronic acid, both the pituitary hormone FSH and the ovarian hormone oestradiol may be influencing where tumour cells home to and survive. The evaluation of the prognostic value of individual hormone levels from AZURE was presented in chapter 3 and showed a low follicle stimulating hormone (premenopausal levels) was borderline significant as a prognostic marker for bone recurrence (Fig 3.5). This suggests that either breast cancer cells have an increased propensity to spread to premenopausal bone, or that the tumour cells that arrive in bone are more likely to survive and subsequently develop autonomous growth and form bone metastases. In addition, a low oestradiol (postmenopausal levels) was borderline significant as a prognostic marker for distant but not bone recurrence (Fig 3.6). This suggests that a postmenopausal bone may not be attractive for tumour cells and therefore they may preferentially spread to alternative distant sites. This differential effect of reproductive hormone levels on tumour cell homing to bone and subsequent tumour growth is supported by work carried out within Sheffield Medical School using an *in vivo* model of bone metastases. 12-week old female balb/c nude mice were injected intracardiac with DiD labeled ER-ve MDA-MB-231 breast cancer cells 7 days post ovariectomy (OVX) or sham procedure. Mice were culled at either 24 hours post tumour cell injection for evaluation of homing of tumour cells to bone, or maintained for 48 days for evaluation of tumour growth in bone. At the 24 hour time-point there was a higher number of tumour cells in bone in sham animals compared to OVX (personal communication from Dr Ning Wang, Academic Department of Human Metabolism, Sheffield University) (Fig 7.2). Serum levels of reproductive hormones were not evaluated in this study, however, sham animals would be expected to have a lower FSH level than OVX animals, since the OVX procedure removes the negative feedback of the ovarian hormones on pituitary FSH secretion, indicating a low FSH at the time of tumour cell injection may increase bone homing. At the 48-day time-point, tumour growth in bone was significantly higher in the OVX than sham group (89% vs. 18%) (personal communication from Dr Penny Ottewell, Academic Department of Clinical Oncology, Sheffield University). These data suggest that ER-ve breast cancer cells home preferentially to sham (premenopausal) bone, but subsequent growth into metastases may be more dependent upon low levels of reproductive hormones (OVX/postmenopausal). Tumour cells outside of bone were not evaluated in this study and therefore data on non-bone tumour growth is not available.





Data kindly provided by Dr Penny Ottewell and Dr Ning Wang, Medical School, Sheffield University.

Figure 7.2 Tumour cell homing to bone in ovariectomised and sham operated mice.

Mice were injected intracardiac with 1x10^5 DiD labeled MDA-MB-231 cells 7 days post sham or OVX procedure. After a further 7 days mice were culled and hind limbs collected for analysis using multi-photon microscopy or flow cytometry of flushed bone marrow. Data represents mean+SEM. Students t test for significance, *=p value <0.05.

The molecular mechanisms of how reproductive hormones such as inhibin A+/ oestradiol could modify the effects of zoledronic acid on DTCs in early breast cancer had not been identified to date. The data presented on the ANZAC trial in chapter 4 showed a novel differential effect of zoledronic acid on serum levels of the TGFβ superfamily of proteins, according to menopausal status. The ANZAC trial was a neo-adjuvant clinical study evaluating the addition of zoledronic acid to first cycle of standard FEC_{100} chemotherapy. Follistatin significantly fell and TGFβ1 significantly increased at day 21 compared to baseline in patients receiving zoledronic acid plus chemotherapy compared to chemotherapy alone (Fig 4.6 + Fig 4.8). The change in follistatin with zoledronic acid was driven by postmenopausal patients, who also had a significant increase in serum activin at day 21 (Table 4.7). These data suggested a new novel anti-tumour mechanism of zoledronic acid via alterations in serum follistatin, activin and TGFβ1. Whether alterations in serum levels of these proteins with zoledronic acid reflected a direct effect on the primary breast tumour secretion of proteins, or an indirect effect on bone secretion could not be determined in this study.

The differential direct anti-tumour effects of zoledronic acid according to menopausal status had been confirmed from other neo-adjuvant clinical studies. NEOZOTAC was a phase III randomized trial of neo-adjuvant taxane based chemotherapy +/- zoledronic acid in HER2 –ve, stage II/II breast cancer patients (n=250). The primary endpoint was pathological complete response (pCR) of the primary breast tumour. pCR results from 228 patients were presented at ASCO 2013 and did not differ between treatment groups, but a trend was observed for an increased pCR in postmenopausal women receiving zoledronic acid (18% vs 11%) (Charehbili A 2013). A further trial in a similar population group randomised patients to neo-adjuvant taxane containing chemotherapy +/- zoledronic acid, and data from 188 patients were presented at ASCO 2013. There was no significant difference in pCR rates with zoledronic acid in the overall population, but a trend to increased pCR rates with zoledronic acid in postmenopausal women (18.4% vs 5.4%) and women with ER-ve tumours (35.3% vs 11.8%) was shown. In patients who were both postmenopausal and had ER-ve tumours, pCR rates were increased with zoledronic acid to a greater degree than in the individual sub-groups (50% vs 0%, p=0.077)(Horiguchi J 2013). These data suggested that both oestrogen receptor status and menopausal status could influence the direct anti-tumour efficacy of zoledronic acid, and they may be synergistic with one another. However, the molecular mechanism of this direct anti-tumour effect remained to be established.

A novel differential direct effect of zoledronic acid on the secretion of activin and follistatin, according to ER status of breast cancer cell lines *in vitro,* was shown in chapter 5. Zoledronic acid decreased follistatin secretion from ER-ve breast cancer cell lines but not ER+ve cell lines (Fig 5.6). This alteration in follistatin secretion had relevant effects on downstream signaling pathways in the ER-ve breast cancer cell line MDA-MB-231, by increasing phosphorylation of the activin receptor associated protein Smad2C (Fig 5.18), a known tumour suppressor in breast cancer. Moreover, activin inhibited proliferation of both cell lines, and this was negated in the presence of follistatin (Fig 5.13). These data suggested that in ER-ve cell lines, zoledronic acid can inhibit proliferation partly by increasing the bioavailability of the tumour suppressors activin and Smad2C, by decreasing the extracellular activin inhibitor, follistatin. This effect was confirmed *in vivo* in a xenograft model of sub-cutaneous ER-ve tumours treated with zoledronic acid or PBS control. A significant decrease in the area of tumour expressing follistatin was shown in mice treated with zoledronic acid compared to PBS control (Fig 5.21 a+b). The ability of zoledronic acid to decrease follistatin secretion from ER-ve MDA-MB-231 cells was diminished if the ovarian hormone inhibin A was added to conditioned medium (Fig 6.3 and 6.4), suggesting that this novel direct anti-tumour effect of zoledronic acid is more pronounced when there are low levels of female hormones in the tumour microenvironment.

These data supported the hypothesis that zoledronic acid could directly affect activin and follistatin secretion from breast cancer cell lines *in vitro*, and this novel effect was influenced by the ER-status of the tumour cells and levels of inhibin A in the tumour microenvironment. Recent data from the final efficacy analysis of adjuvant zoledronic acid in the AZURE trial (Coleman RE 2013) showed a synergistic improvement in invasive disease free survival outcomes, in bone and outside of bone, in patients who were both > 5 years postmenopausal and had ER-ve breast tumours (HR 0.687; 95% CI 0.474-0.996) (Fig 7.3). In the adjuvant setting, when primary breast tumours have been surgically removed, the anti-tumour effect of zoledronic acid is likely to be indirect, via modification of the bone microenvironment, with subsequent effects on DTCs that have

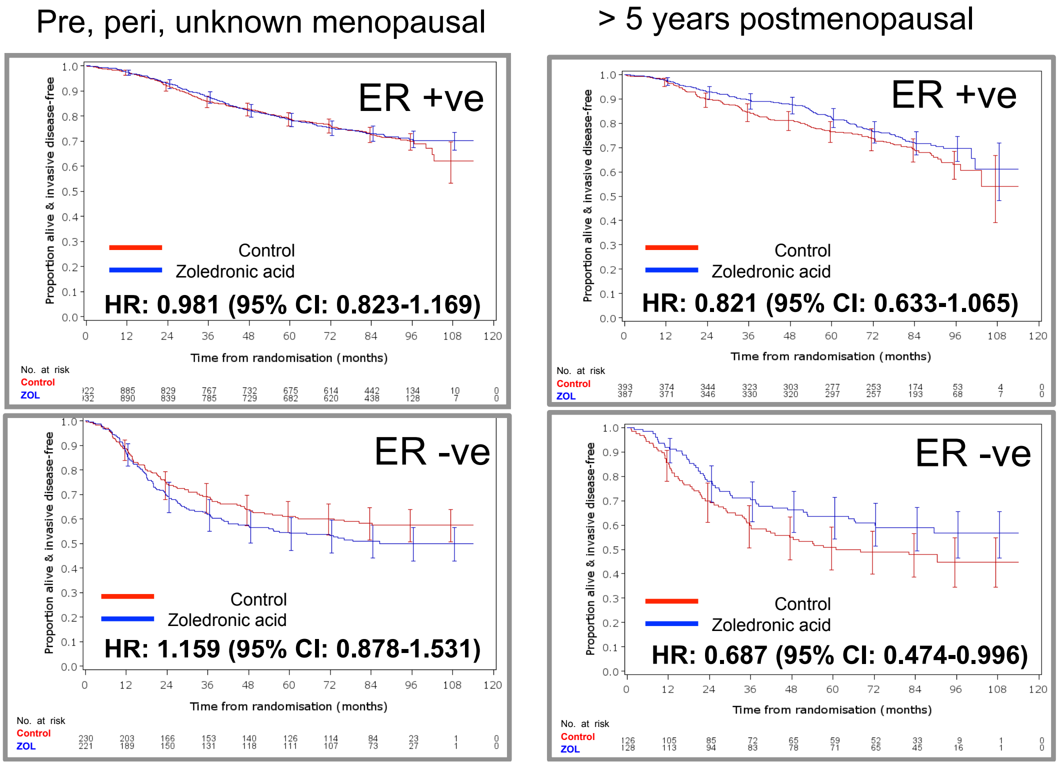


Figure reproduced with permission from RE Coleman. Adjuvant therapy for stage II/III breast cancer with or without zoledronic acid. Final efficacy analysis of the AZURE trial. Presented at Cancer and Bone Society, Miami, Nov 2013. Abst; LBA001

Figure 7.3 Effect of both ER status and menopausal status on invasive disease free survival outcomes with addition of zoledronic acid to standard therapy

Women > 5 years postmenopausal and with ER-ve tumours derived the most benefit from adjuvant zoledronic acid with a higher proportion of women being alive without invasive disease recurrence at 120 months follow up (HR 0.687, 95%CI 0.474-0.996).

spread to bone. These results indicated that zoledronic acid's indirect anti-tumour effect was also influenced by the ER status of the DTCs located in bone as well as levels of reproductive hormones. Data from the ANZAC study (chapter 4) showed changes in serum levels of follistatin post zoledronic acid treatment, were also affected by ER-status of patients primary breast tumours. There was a significant reduction in serum follistatin secretion at day 5-post zoledronic acid plus chemotherapy compared to those receiving chemotherapy alone in patients with ER-ve tumours only (Table 4.8). As discussed previously these changes in serum levels could not be attributed to either changes in the primary tumour secretion or alternative sources such as bone. It was therefore necessary to evaluate if zoledronic acid could alter the levels of activin and follistatin in the bone microenvironment.

The data from chapter 6 showed a novel indirect affect of both zoledronic acid and inhibin A on activin and follistatin levels in the bone microenvironment *in vivo*. Zoledronic acid decreased levels of the activin inhibitor follistatin in bone, but was only able to do so in the absence of ovarian hormones i.e. animals that had undergone ovariectomy (Fig 6.22). Exogenous recombinant inhibin A decreased activin levels in the bone (Fig 6.18). This may create two different bone environments that DTCs would be exposed to; an inhibin A rich + low activin environment (modeling premenopausal) and a low inhibin A + activin rich environment (modeling postmenopausal). Addition of zoledronic acid in the low inhibin environment would be expected to further increase activin's bioavailability by decreasing its inhibitor follistatin. These results suggest that zoledronic acid may have differential effects on bone activin:follistatin levels in the presence or absence of inhibin A. The data presented in chapter 6 however, did not confirm whether inhibin A was the primary ovarian hormone responsible for the interaction with zoledronic acid, since ovariectomy will remove the biological effects of both inhibin A and oestradiol. Oestradiol has well documented anabolic effects in bone (Nicks, Fowler *et al*. 2010). To assess the affect of oestradiol on bone levels of activin and follistatin, calvaria were collected from 12-week old balb/c nude mice that underwent OVX on day 1 of experiment and sub-cutaneous oestradiol pellets (1.5mg) were inserted on day 5, with mice maintained for 28 days (in vivo work carried out by Dr Penny Ottewell). Oestradiol had a profound effect on bone volume and significantly decreased activin levels in bone with a concurrent increase in follistatin (Fig 7.4). This novel effect suggested that both inhibin A and oestradiol may influence the bioavailability of the

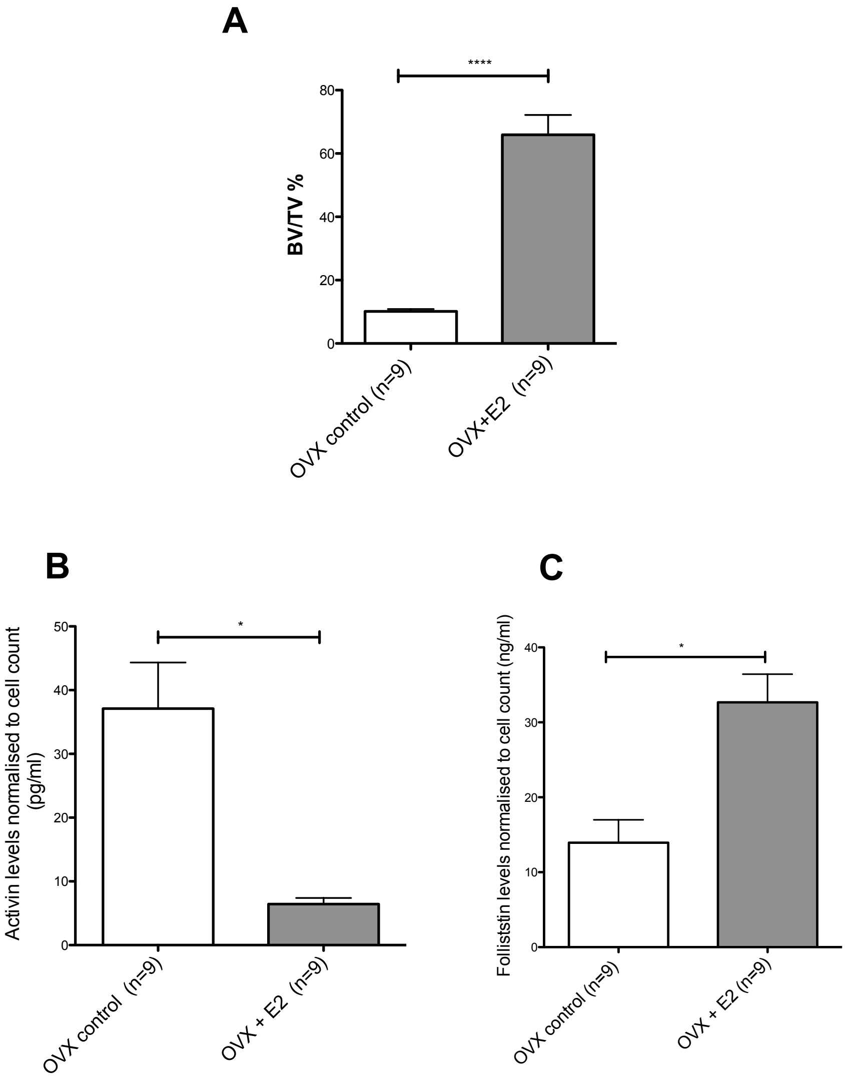


Figure 7.4 Effect of oestradiol (E2) on bone volume and bone levels of activin and follistatin in ovariectomised mice.

12-week old balb/c nude female mice underwent ovariectomy on day 1 with insertion of sub-cutaneous oestradiol pellets (1.5mg) on day 5. Mice were maintained for 28 days and calvaria were collected at termination of experiment, crushed in PBS, filtered and supernatant processed to activin or follistatin ELISA. **A.** Change in bone volume **B** Change in activin levels in calvaria, **C**. Change in follistatin levels in calvaria. Data represents mean +SEM. ****p value <0.0001, * p value <0.05.

tumour suppressor activin in bone. This may have an effect on DTCs, in particular ER-ve DTCs, in the bone microenvironment and requires further investigation.

Future work is planned to investigate the effects of inhibin A and zoledronic acid on ER-ve (MDA-MB-231) tumour cell growth in bone using established *in vivo* models of breast cancer metastases to bone, in conjunction with the recombinant inhibin A used in this thesis. This will assess if the effects of inhibin A and zoledronic acid on activin and follistatin levels within the bone microenvironment (discussed in chapter 6) can indirectly affect ER-ve tumour cell growth in bone. In addition, to further characterize the direct effect of zoledronic acid on follistatin secretion from breast tumours, primary breast tumour tissue blocks from the neo-adjuvant ANZAC study (FEC$_{100}$ chemotherapy +/- zoledronic acid at first cycle) will be evaluated for follistatin expression using the optimized follistatin antibody used in chapter 5, comparing baseline with day 5 and day 21 breast biopsies. Identification of the source of serum follistatin in breast cancer patients would assist in understanding if tumour cell secretion or bone secretion of follistatin were driving the changes in serum levels seen in postmenopausal patients in the ANZAC study. To assess this, serum from breast cancer patients receiving zoledronic acid with adjuvant chemotherapy would be an ideal resource for comparison, and could potentially be evaluated if adjuvant zoledonic acid becomes standard therapy in postmenopausal breast cancer patients.

In summary, the data presented in this thesis has shown a novel direct anti-tumour mechanism of zoledronic acid in ER-ve breast cancer cells both *in vitro* and *in vivo,* by decreasing follistatin secretion from tumour cells and thus increasing bioavailability of the tumour suppressor activin. The ability of zoledronic acid to affect follistatin secretion from tumour cells was modified by the presence of the ovarian hormone inhibin A *in vitro*. Zoledronic acid may also have an indirect effect on DTCs in the bone microenvironment that is enhanced in the presence of low levels of ovarian hormones, with evidence *in vivo,* that zoledronic acid decreased follistatin levels in bone, only in OVX animals. Moreover, serum follistatin levels in neo-adjuvant patients receiving a single treatment of 4mg IV zoledronic acid significantly fell at day 21 compared to baseline in postmenopausal women, and at day 5 in patients with ER-ve breast tumours, suggesting zoledronic acid may be modifying secretion of the protein into serum. Although no single reproductive hormone was identified to be a significant predictor of response to adjuvant zoledronic acid in the AZURE trial analysis, these data together suggest that ovarian hormones can interact with zoledronic acid to affect the activin:follistatin signaling proteins in both the tumour and in the bone microenvironment, with potential implications for tumour cell proliferation and survival.

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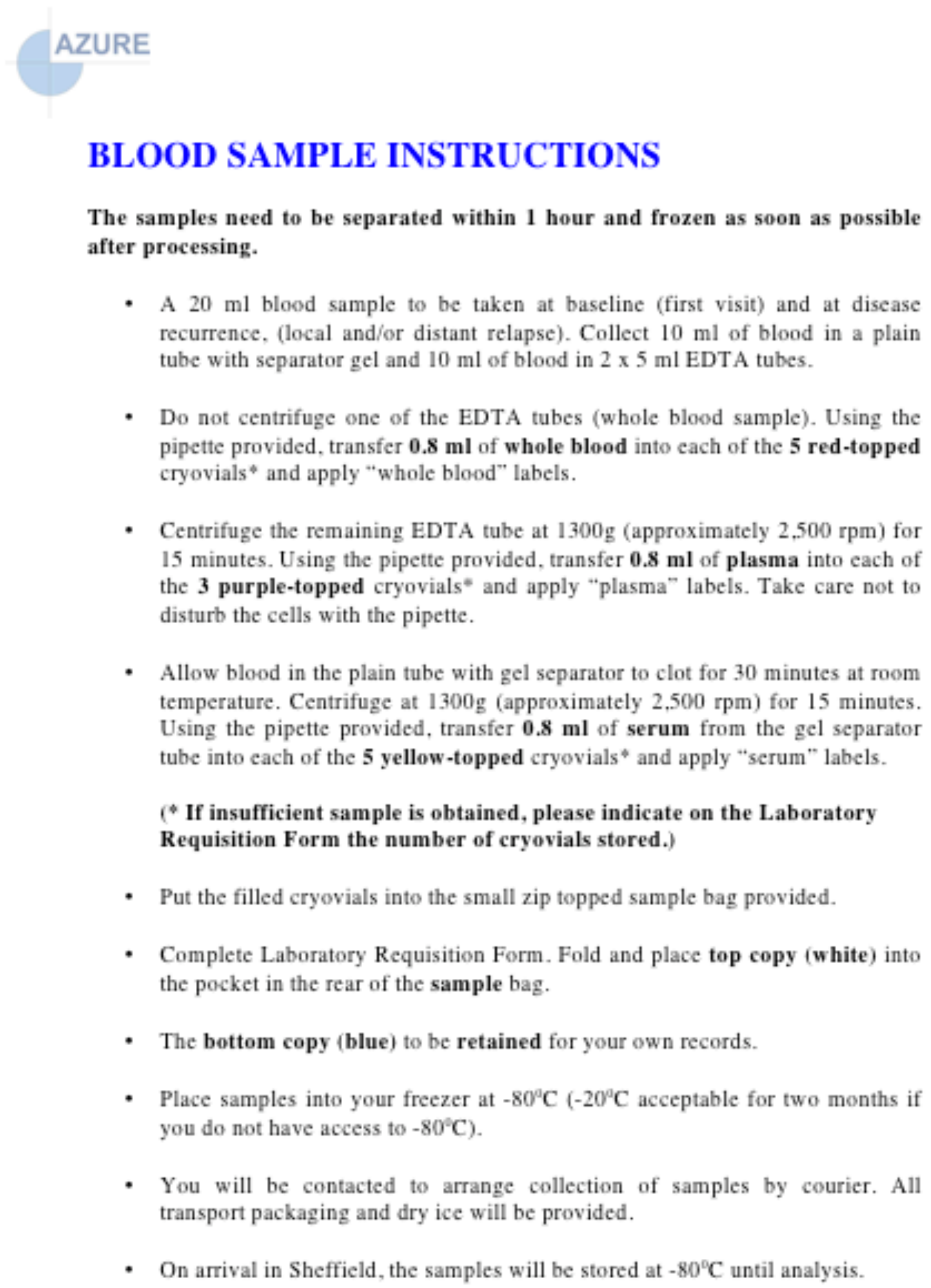
Appendix.

Appendix 1. AZURE laboratory manual for collection of serum



AZURE

BLOOD SAMPLE INSTRUCTIONS

The samples need to be separated within 1 hour and frozen as soon as possible after processing.

- A 20 ml blood sample to be taken at baseline (first visit) and at disease recurrence, (local and/or distant relapse). Collect 10 ml of blood in a plain tube with separator gel and 10 ml of blood in 2 x 5 ml EDTA tubes.
- Do not centrifuge one of the EDTA tubes (whole blood sample). Using the pipette provided, transfer **0.8 ml** of **whole blood** into each of the **5 red-topped** cryovials* and apply "whole blood" labels.
- Centrifuge the remaining EDTA tube at 1300g (approximately 2,500 rpm) for 15 minutes. Using the pipette provided, transfer **0.8 ml** of **plasma** into each of the **3 purple-topped** cryovials* and apply "plasma" labels. Take care not to disturb the cells with the pipette.
- Allow blood in the plain tube with gel separator to clot for 30 minutes at room temperature. Centrifuge at 1300g (approximately 2,500 rpm) for 15 minutes. Using the pipette provided, transfer **0.8 ml** of **serum** from the gel separator tube into each of the **5 yellow-topped** cryovials* and apply "serum" labels.

  (* If insufficient sample is obtained, please indicate on the Laboratory Requisition Form the number of cryovials stored.)
- Put the filled cryovials into the small zip topped sample bag provided.
- Complete Laboratory Requisition Form. Fold and place **top copy (white)** into the pocket in the rear of the **sample** bag.
- The **bottom copy (blue)** to be **retained** for your own records.
- Place samples into your freezer at -80°C (-20°C acceptable for two months if you do not have access to -80°C).
- You will be contacted to arrange collection of samples by courier. All transport packaging and dry ice will be provided.
- On arrival in Sheffield, the samples will be stored at -80°C until analysis.

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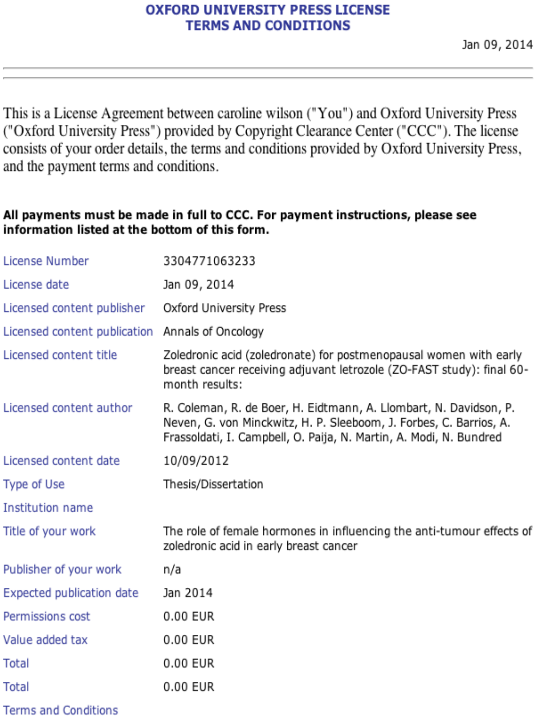

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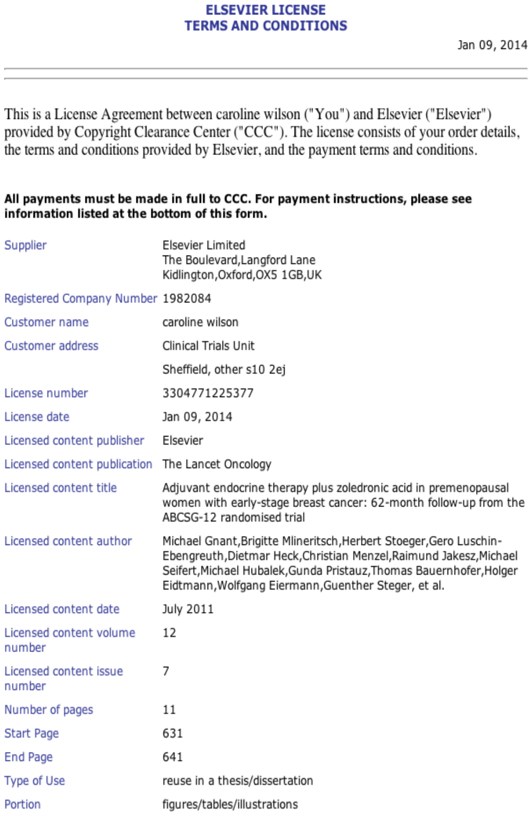

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